THE EFFECT OF BIOSTIMULANTS ON THE PERFORMANCE OF NEWLY PLANTED APPLE TREES IN FUMIGATED SOIL

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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DEDICATION

I dedicate this thesis to the following:

- Firstly, to our Heavenly Father
- Eben & Teresa van der Wath
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- Dr. Elmi Lötze
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SUMMARY

Apple replant disease (ARD) is a consequence of replanting apple orchards in suitable climatic areas with limited availability of virgin soils. Fumigation of ARD infected soils provides alleviation of the disease pressure. However, the influence thereof on the dynamics of natural occurring beneficial soil microbe communities has become an environmental concern. Documentation of the addition of biostimulants for sustainable rehabilitation of the soil microbial community after fumigation and enhancement of tree performance is limited under South African conditions.

The aim of this study was to evaluate the effect of biostimulants on the performance of young, non-bearing apple trees, planted in fumigated soil, under local conditions. The objectives were to determine: i) the effect of biostimulants on root growth dynamics quantified with destructive and non-destructive measurements, ii) investigate the effect of biostimulants on the microbial colonization of tree roots and iii), the effect of biostimulants on aerial vegetative parameters and physiology.

Treatments included Mycorrhiza, three different formulations of *Trichoderma* based products (Trich 1 & Trich 2), L-AA, Plant Extract, Compost and an untreated Control. The experiment was conducted on Lovenstein farm, Vyeboom, in the Western Cape on 'Granny Smith/MM109' trees in a randomised complete block design.

Paper 1 showed significant differences between treatments for root dynamics, occurring primarily in the top layers of the soil, for both destructive and non-destructive parameters. During the first season, the lowest total root number and total root length were observed in the Mycorrhiza treatment, while Trich 1 and Trich 2 treatments showed the highest values. Seasonal trends showed that the Mycorrhiza treatment had the most prominent effect on total root area and total root volume.

Significant differences between treatments regarding the total root number were reported in Paper 2, with treatments Trich 1 and 2 differing significantly from all other treatments. Additionally, possible antagonism between *Trichoderma spp.* and sclerotic bodies in the Compost and Trich 2 treatments was also observed.

No significant differences were observed between treatments in Paper 3, neither for stress remediation nor vegetative, aerial growth. The effect of biostimulants on plant performance varied according to parameters used for quantification and product. This supported current literature reporting a lack of consistency and inconclusive results when biostimulants are applied in the field. It also indicated the importance of knowledge about the mode of action of biostimulants and the best time of intervention, in addition to selecting the correct equipment for the just phenological stage to capture plant responses after application. Nevertheless, significant differences between selective treatments in Paper 1 suggest that an adaption of the protocol may lead to more conclusive differences when repeating the study.

OPSOMMING

Appelhervestigingsiekte (AHS) is die gevolg van die hervestiging van appelboorde in geskikte klimaatsareas met beperkte beskibaarheid van maagdelike grond. Beroking van AHS geïnfekteerde grond verskaf ‘n opsie om siektedruk te bestuur. Daarteenoor staan die groeiende bekommernis rakende die effek daarvan op die dinamika van natuurlike voordelige mikrobe-gemeenskappe in die omgewing. Dokumentasie oor die toediening van biostimulante vir die volhoubare rehabilitasie van grondmikrobe-gemeenskappe en die verhoging van boomprestasie is beperk onder Suid-Afrikaanse omstandighede.

Die doel van hierdie studie was om die effek van biostimulante op die prestasie van jong, nie-draende appelbome, geplant in ‘n berookte grond, onder plaaslike toestande, te evalueer. Die oogmerke was om te bepaal: i) wat die effek van biostimulante is op wortegroeidinamika, gekwantifiseer deur destruktiewe en nie-destruktiewe metings, ii) ‘n ondersoek na die effek van biostimulante op mikrobe kolonisasie van boomwortels en iii), die effek van biostimulante op bogrondse vegetatiewe parameters en fisiologie.

Behandelings het die volgende behels: Mikorrhizae, drie formulasies van *Trichoderma* gebaseerde produkte (Trich 1 & Trich 2), L-AA, Plantekstrak, Kompos en ‘n onbehandelde Kontrole. Die eksperiment is uitgevoer op Lovenstein, Vyeboom, in die Weskaap op ‘Granny Smith/MM109’ bome in ‘n volledig, gerandomiseerde blok ontwerp.

Artikel 1 het betekenisvolle verskille tussen behandelings aangedui vir worteldinamika wat primêr in die boonste grondlae voorgekom het, in beide die destruktiewe en nie-destruktiewe parameters. Gedurende die eerste seisoen, is die laagste aantal wortels en totale wortellengte opgemerk in die Mikorrhiza behandeling, terwyl behandelings Trich 1 en Trich 2 die hoogste waardes getoon het. Seisoenale tendense het getoon dat die Mikorrhiza behandelings die prominentste effek op die totale wortelarea en totale wortelvolume gehad het.

Betekenisvolle verskille tussen behandelings ten opsigte van die totale aantal wortels is gerapporteer in Artikel 2, met behandelings Trich 1 en 2 wat betekenisvol verskil het van die res van die behandelings. Bykomend, is ‘n moontlike antagonisme tussen *Trichoderma* spp. en sklerosia liggame in die Kompos en Trich 2 behandelings waargeneem.

Geen betekenisvolle verskille is waargeneem tussen behandelings in Artikel 3 vir stres verligting of vegetatiewe, bogrondse groei nie. Die effek van biostimulante op plantprestasie het verskil afhangende van die parameter wat gebruik is vir kwantifisering en die produk. Dit het bestaande literatuur bevestig wat berig het oor die wisselvalligheid en onvolledige resultate wanneer biostimulante in die veld toegedien word. Dit dui ook op die belangrikheid van kennis rakende die metode-van-aksie van biostimulante en die beste tydstip van ingryping, ter aanvulling van die keuse van die korrekte toerusting en bepaling van die juiste fenonologiese fase om die plantreaksie na

toediening, vas te lê. Desondanks het die betekenisvolle verskille tussen spesifieke behandelings in Artikel 1 aangedui dat ‘n aanpassing in die protokols mag lei tot meer konkrete verskille indien die studie herhaal sou word.

This thesis is a compilation of chapters, starting with a literature review, followed by three research papers. Each paper is prepared as a scientific paper using the format of the *Southern African Journal for Plant and Soil*. Repetition or duplication between papers is therefore unavoidable.

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GENERAL INTRODUCTON

Apple production in South Africa is limited to specific regions due to the temperate climate requirement of this crop (Du Plessis and Schloms, 2017). Together with the limited availability of virgin soils for expansion, an increased demand for apples (HortGro, 2019; Fruit SA, 2018) led to replanting of new orchards on previously cultivated sites (van Schoor *et al.*, 2009). This presents a challenge, as newly planted trees are predisposed to disease pressure caused by a complex of pathogens in the soil (Mahnkopp *et al.*, 2018), which leads to a decline in tree performance, yield and ultimately death of the trees (Bahilu *et al.*, 2016). This phenomenon is referred to as apple replant disease (ARD) (Rumberger *et al.*, 2004).

The effective control of ARD with chemical fumigation has often been reported (Noling, 2008; Nyoni *et al.*, 2019; Winkelmann *et al.*, 2019). The negative impact of the chemicals used in fumigation on the environment, the balance in soil microbiology and on humans (Maluin *et al.*, 2020), led to the search for alternative measures to control ARD (van Schoor *et al.*, 2009). By restoring beneficial soil microbiology to its natural state after fumigation, an environment favourable for the growth of young apple tree roots will be created (Egamberdieva *et al.*, 2017). It may also reduce the effect of transplant shock, pathogenic infection and abiotic stress in establishment and growth of the young tree (Winkelmann *et al.*, 2019).

The use of biostimulants has been studied in more depth during recent years (Rouphael *et al.*, 2020). The understanding of the function and mode of action of biostimulants is a complex field of study and not yet clearly defined for all products (Calvo *et al.*, 2014; du Jardin, 2015; Ricci *et al.*, 2019; Rouphael *et al.*, 2020; Sharma *et al.*, 2014). Claims of the various biostimulant products include enhancing plant nutrient uptake, alleviating abiotic stress and inducing a systemic response within the plant that leads to a tolerance to biotic stress (du Jardin, 2015; Ricci *et al.*, 2019; Rouphael *et al.*, 2020). However, many of these claims are based on results obtained from *in-vitro* studies (Henfrey *et al.*, 2015). Conflicting results, especially for field studies, were reported in various parts of the world. Field studies are difficult to perform due to the normal variability in plant material and environmental conditions. The lack of reliable, consistent results on biostimulant performance from field studies were accentuated in the recent publication by Rouphael *et al*. (2020).

The aim of this study was therefore to investigate some of these claims, by using selected, local commercial biostimulants in a field study, using newly planted ‘Granny Smith’ apple trees in fumigated (1,3-dichloropropene) soils, under South African conditions. Biostimulants from different biostimulant classes were selected: Control (no product), Compost, Mycorrhizae, *Trichoderma* as various combinations (Bio-Tricho Liquid, Excalibur Gold and Aminostim.Xtra®) and a Plant extract (Aqua Clean S.A. - Sludge Abate). Evaluations of treatments were performed as follows:

- Quantifying the effect of treatments on root growth dynamics using i) non-destructive, in-situ minirhizotron images throughout the season and ii), two-dimensional, destructive root analyses at the termination of the study.
- Evaluating the effect of treatments on microorganisms nine months after application, using root colonization of *Trichoderma* spp. as a biological indicator of soil-health recovery and relating results to root growth dynamics.
- Evaluate the effect of treatments on plant performance above ground, quantified by selective physiological stress indicators, as well as vegetative growth of newly planted 'Granny Smith' apple trees in fumigated soil.

The importance and originality of this study pertains to the exploration of biostimulant reactions under field conditions, using products from different biostimulant groups, addressing biotic and abiotic stresses that typically occur during establishment of apple trees after fumigation. This study investigates whether biostimulants can be a sustainable alternative to rehabilitate soil microbial populations after chemical fumigation, which should yield more insight towards the viability of using these formulations of biostimulants under field conditions for sustainable farming in the future.

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LITERATURE REVIEW

The Role of Soil Fumigation in Apple Orchard Establishment

1. INTRODUCTION

According to Fruit Stats SA (2018), South Africa ranks at the 15th position internationally, from 2010 to 2019, by exporting on average about 45% of the total volume produced globally (HortGro, 2019; Fruit SA 2018). Apples sold on the export market generate a greater unit price than that achieved on the local market, thus making it economically important for producers to achieve high yields of good quality to meet export demands and standards (Department of Agriculture Forestry and Fisheries, 2012).

Due to the high biodiversity linked to the main apple production areas, particularly that of the Western Cape region, the establishment of new apple orchards in virgin soils is becoming increasingly more limited in South Africa. Thus, the need to re-use previously cultivated soil has become a standard practice (van Schoor *et al.,* 2009). When replanting on soils that formerly contained deciduous fruit orchards, the risk of soil borne pathogens and the predisposition of the rhizosphere to disease pressure is much higher than when orchards are established on maiden soils (Bahilu *et al.,* 2016). Therefore, prior to planting, standard procedures are followed such as soil testing (Nemlab, Klapmuts, South Africa) to determine, and if required, treat potential pest and disease threats (Yang *et al.,* 2012) including, but not limited to nematodes (Tewoldemedhin *et al.,* 2011) and *Rhizoctonia* infestation (Tewoldemedhin *et al.,* 2011; van Schoor *et al.,* 2009; York *et al.,* 2018).

The chemical balance of existing fruit orchard soils are considered disturbed, due to years of selective fertiliser application (Lin *et al.,* 2019), herbicide sprays (Heydari and Misaghi, 2011) and other standard management practices such as tillage (Bahilu *et al.,* 2016). Soil mineral analysis is an effective tool to evaluate the soil organic matter status and identify possible mineral deficiencies. This can be amended before replanting and serves as a basis for recommendations for amelioration of physical soil conditions such as soil compaction or water logging, amongst others. However, another challenge may arise when replanting an orchard site with the same crop as before. In the case of apple orchards, it is called Apple Replant Disease (ARD) (Rumberger *et al.,* 2004; Yao *et al.,* 2006). ARD as defined by Mahnkopp et al. (2018) is the alteration of the microbiome of soils due to the re-use of soils previously planted with apple cultures, leading to harmful physiological and morphological reactions in the next generation of apple trees planted on the same site. Thus, old orchard sites on which fruit trees such as cherries and apples have been cultivated profitably before, and then replanted

with the same fruit crop, without specific soil treatments, delivered suboptimal yields, and are considered to have ARD (Mai *et al.,* 1994).

However, not all apple trees that are re-planted are affected equally by ARD (van Schoor *et al.,* 2009). Factors associated with ARD include both biotic (biological) and abiotic (physical) stresses such as the loss of soil organic matter, limited nutrient availability, chemical residues of herbicides and non-optimal soil structure (Mai *et al.,* 1994), where the impact and the severity of these factors may vary for each region-site combination. ARD symptoms include water stress and nutrient deficiencies, both which are physiological conditions that may result in necrosis of feeder roots and cause stunted above- and below-ground tree growth (Rumberger *et al.,* 2004). The effects of orchard replant disease (ORD), a condition like ARD, may also lead to low tolerance for stress factors. In New York, replanted cherry orchards that suffered from ORD were also associated with a higher susceptibility to cold injury during winter months (Mai *et al.,* 1994).

ARD is currently controlled by mainly using semi-selective chemicals in pre-plant soil fumigation (Nyoni *et al.,* 2019). This means that the effect of singularly formulated chemicals do not affect a broad range of targeted plant parasitic microorganisms within the soil as it would when using in combination with other chemicals or as seen with the use of a broad spectrum fumigant such as Methyl Bromide (MB). A broad description of soil fumigation is given by Tewoldemedhin et al. (2011) as "a pre-plant chemical treatment of soil, using a pesticide product that converts to form a volatile gas". Control of most soil borne disease and pathogens is obtained as the diffusible fumigation gas moves through the pore space of the soil (Noling, 2008). However, gasses used to fumigate the soil may also be phytotoxic to plants and therefore first need to dissipate from the soil before replanting can commence, in order to avoid crop injury (Maluin *et al.,* 2020). Fumigants are applied to annual and perennial crop sites either as a single treatment (effective to a lesser extend), or in combination with each other, to obtain control over a wide range of soil inhibiting species as "multi-purpose" or "broad-spectrum" fumigants (Winkelmann *et al.,* 2019).

The causative agents of ARD, together or individually, can broadly be narrowed down to soil bacteria (*Bacillus* and *Pseudomonas*), plant parasitic nematodes (*Paratylenchus* spp.), selected fungi (*Cylindrocarpon* and *Rhizoctonia*), actinomycetes and oomycetes (*Pythium* and *Phytophthora*) (Cabrera *et al.,* 2015; Gao *et al.,* 2016; Jaffee *et al.,* 1982; Nicola *et al.,* 2017; Rumberger *et al.,* 2007; Tewoldemedhin *et al.,* 2011; Wilson *et al.,* 2004; Yao *et al.,* 2006). Abiotic (physical) factors such as the loss of soil organic matter, limited or excessive nutrient availability, chemical residues of herbicides and soil structure are known to make a major contribution to the extent and intensity of ARD occurrence (Mai *et al.,* 1994; Yao *et al.,* 2006). Therefore, effective control of ARD is a major challenge. Even though soil fumigation was shown to improve tree growth by limiting ARD, this may

not be true for all sites and circumstances (Nicola *et al.,* 2017). Thus, there is an urgent need for more fumigation products and/or alternative solutions to control ARD.

The harsh impact of the chemical control of ARD on the environment and the possible effects on human health has initiated numerous studies to find alternative remedies to provide control of ARD (van Schoor *et al.,* 2009). Such interventions include compost and organic matter amendments that were successful in suppressing the disease on a commercial scale (Hoitink *et al.,* 1997). Another approach of interest is the use of brassicaceaous seed meal formulations that proved effective to alleviate disease symptoms caused by ARD in conventional and organic systems, although it showed limitations when applied as a single treatment (Mazzola and Brown, 2010).

The aim of this review is to discuss the effect of soil fumigation on orchard establishment as a horticultural practice within the commercial horticulture sector, with specific focus on fruit tree crops. Both advantages and disadvantages will be evaluated to include the short- and long-term effects of fumigation on the performance of the affected crop, as well as on dynamics of soil microbial populations. A more integrated understanding of the interactions between biotic and abiotic factors within an orchard micro-biome will contribute significantly towards the knowledge base required for future sustainable food production, especially in the light of recent and still eminent withdrawal of the registration of key soil fumigant products.

2. THE IMPORTANCE OF FUMIGATION

In high value cropping systems, such as commercial apple orchards, fumigants are used to protect such major investments as opposed to accepting serious economic losses that can be incurred without the use thereof (Gao *et al.,* 2016). This approach was supported by van Schoor et al. (2009) who reported that fumigation improved growth in three-month-old potted 'Golden Delicious' apple seedlings compared to the untreated control seedlings in replanted soil. However, the effect of fumigation, which may be positive and/or negative on the growth of the subject crop, are dependent on numerous factors which will be discussed throughout this review.

In South Africa, the Western Cape Province is the primary production area and accounts for more than half of all the apples produced in South Africa (Fruit Stats SA, 2019). The climate of the Western Cape region is characterized by hot, dry summers, with its rainfall mainly concentrated in winter, typical of a Mediterranean climate (Du Plessis and Schloms, 2017). The soil is nutrient poor and consists of a low percentage organic matter, as well as a low cation exchange capacity (CEC) (van Schoor *et al.,* 2009). In addition to these pre-existing soil limitations, ARD poses an additional challenge in the establishment of apple orchards on replanted soil. ARD may impact to such an extent that the profitability of an apple orchard can be decreased by 50% throughout its lifespan (van Schoor

et al., 2009), with particularly prominent effects in young trees, during the establishment years of the orchard. Therefore, fumigation before replanting is a standard practice in South African apple orchards. However, the new challenges in future would be to find suitable alternative fumigants and/or practices to replace the genotoxic (Motawei and Abdel-Salam, 2017), but highly efficient methyl bromide (MB), with more environmentally friendly alternatives to promote sustainable agricultural practices.

i. Effectiveness of fumigation

The effectiveness of soil fumigation in apple orchard establishment is dependent on a number of factors ranging from an optimal ambient temperature (to a lesser extent), soil temperature and soil moisture content at the time of application, to the effects of soil texture, duration of exposure of the organisms to fumigation and the concentration of fumigant applied (Menge, 1982). López-Aranda *et al*. (2016) showed that fumigants used in combination, such as chloropicrin (CP) and 1, 3 dichloropropene (1, 3D) in a concentration of 50:50 (w/w), produced optimum results in controlling weeds in strawberry nurseries, as opposed to using only CP. Similarly, for the highest efficacy of ARD control, standard procedures for the application of MB included soil temperatures of 17 ˚C or higher at a depth of 100 mm, and a soil moisture content that would allow seed germination, but still being sufficiently dry to allow for sufficient penetration of the fumigation gas (South African Bureau of Standards Code of Practice 0204, 1998). In commercial nurseries of perennial fruit and nut trees, 1, 3-D was an approved treatment on sandy soils, however on soils with a finer texture, the adequate control of pests was not achieved (Duniway, 2002). Thus, also indicating the sensitivity of fumigants to soil texture in addition to previous mentioned factors.

ii. The direct and indirect effect of chemical soil fumigation products

The response of a crop to fumigation cannot only be attributed to the elimination of harmful microorganisms and soil-borne pathogens (Bailey and Lazarovits, 2003). Fumigation responses may also be affected by other factors such as the release of nitrogen and other metabolites from the microorganisms when eliminated by the treatment (Magarey, 1999). The use of CP has been shown to result in an increase of N_2O production as a by-product of dissolved amino acids, or the increase can be linked to microbial biomass nitrogen, as well as emergence as end products of the processes of nitrification and denitrification (Fang *et al.,* 2018a).

In a similar study, the presence of nitrogen-fixing bacteria, including, but not limited to *Rhizobium*, increased after fumigation using Dazomet (DZ) (Fang *et al.,* 2018a; Mahmud *et al.,* 2020), although this phenomenon was partially attributed to the nitrogen emissions which correlated with environmental factors such as microbial biomass rather than the active fixation of nitrogen.

However, the modes of action of most fumigants that are directly associated with the decline in beneficial microorganisms such as mycorrhizal fungi, is due to its direct toxic effect on microbial life (Menge, 1982). These toxic effects included the inhibition of spore development and germination, and limited the effective re-establishment of beneficial organisms such as mycorrhiza. In contrast, *Trichoderma* spp. survived autoclaving and were still effective to result in an increase in plant growth thereafter (De Los Santos *et al.*, 2003). This illustrated that some microorganisms are less susceptible or more resilient to fumigation than others. Furthermore, it was discerned that the selectiveness of the chemicals applied during fumigation is not only dependant on the specific formulation of the chemical applied.

iii. Method of application of chemical soil fumigation products

With conventional applications, soil fumigants were diffused into the soil in a gaseous form through dripper lines that were buried, or by using pressure to inject the fumigant into the soil through shanks mounted on tractors (Duniway, 2002). However, recently the use of emulsified formulations registered for 1, 3-D, can be applied through water delivery, using drip irrigation systems (Schneider *et al.*, 2009). Irrespective of the application method of the fumigant to the soil, a tarp made of polyethylene (PE) or high density polyethylene (HDPE) is used to cover the treated soil, thus ensuring a slower rate of diffusion of the fumigant from the soil to the atmosphere. This also allows a more timely exposure of the soil profile to the gas and therefore increasing the potential control over soil borne pest and pathogens (Schneider *et al.*, 2009).

3. THE EFFECT OF FUMIGATION ON APPLE TREE PERFORMANCE

Chemical fumigation with methyl isothiocyanate (MITC) (Huang *et al.*, 2019), the active ingredient in dazomet (DZ), has shown to enhance plant performance of replanted sites with ARD (Nicola *et al.*, 2017). ARD affected orchards require fumigation before replanting to overcome the negative effects on establishment, initial growth and yield due to the residing pathogen complexes (Menge, 1982). However, even if applied at the optimum time and recommended application rates, fumigation may not always be effective (Yao *et al.*, 2006). With the use of an alternative fumigant, Telone which consist of 78% dichloropropene and 17% chloropicrin, the microbial community of the fumigated soil was altered, yet it did not result in an increase in tree growth or yield (Yao *et al.*, 2006). It is important to note that the particular apple rootstocks used during their trial may have contributed to the lack of growth and yield increase that was reported, despite soils being fumigated. The effect of fumigation on apple tree performance is influenced by various factors, including the type of fumigant used, the apple rootstock, site-region specific climates and soil conditions, particularly soil moisture and texture (Fang *et al.*, 2018b; Nicola *et al.*, 2017; Rumberger *et al.*, 2004).

4. EFFECT OF FUMIGATION ON SOIL BIOLOGY

In addition to the effect of fumigants to eliminate soil-borne pathogens and improve plant performance in newly established apple orchards with ARD, fumigation also had an impact on beneficial microbial communities (Zhang *et al.,* 2019). Nicola *et al.* (2017) proved that the fumigation with Dazomet reduced ARD symptoms, as well as modified the soil microbial communities, by increasing the presence of specific beneficial microorganisms that acted as a biocontrol mechanism against plant pathogens, which was a result of recolonization of beneficial microorganisms within the soil, after fumigation.

Menge (1982) observed that mycorrhizal growth and colonization of roots increased from fumigation or sterilized soil compared to that of mycorrhizae in association with plants cultivated in unsterilized soil. A possible explanation for this that fumigation most likely removed competing pathogens, therefore encouraging growth of beneficial organisms. This hypothesis is also supported by findings of Mehta and Bharat (2013) that reported the positive effect of arbuscular mycorrhiza fungi (AMF) on apple seedlings, specific with regard to the increase in phosphorus (P) uptake in soil fumigated with formaldehyde. Furthermore, the integrated approach of soil fumigation with formaldehyde, together with the use of a bio-control method and a suitable rootstock resulted in a positive beneficial correlation with soil biological activities when rootstock seedlings were planted in fumigated soil (Singh *et al.,* 2017).

The negative effect of fumigants on soil microbiology differs between the various fumigants, with dosage and method of application, between site-region specific environments and differential survival robustness of both beneficial and pathogenic organisms, following fumigation (Fang *et al.,* 2018a, 2018b; Zhang *et al.,* 2019). In an incubation experiment using CP, 1,3-D and meta sodium (MS) fumigants, Sun *et al*. (2020) reported an overall decrease in microbial carbon with fumigation with MS, although no impact on microbial diversity was reported. CP-fumigated soil rapidly recovered its microbial diversity whereas 1,3-D-treated soils showed no rehabilitation of microbial diversity towards the termination of their experiment. These findings support the fact that the effect of fumigation on soil biology is largely dependent on the type of fumigant used, which may show a targeted efficacy toward particular pathogenic and beneficial microorganisms.

5. SOIL FUMIGATION ALTERNATIVES

The search for alternative measures to chemical fumigation for ARD control is driven by the retraction of MB for commercial use, and reported incidences of induced stunted growth after the use of chemical fumigation (Menge, 1982; Noling and Becker, 1994), along with growing concerns about the potential hazardous effects of chemical fumigation on human health and the environment.

Therefore, there is a strong drive towards a more sustainable food production (van Schoor *et al.*, 2009).

i. Chemical fumigants

The use of MB as a fumigant showed satisfactory results in controlling ARD for many years (Ajwa *et al.*, 2010; Mazzola and Mullinix, 2005). In one case, the relationship between natural occurring *Trichoderma* and MB soil fumigation led to higher production in strawberries, with the level of pathogens detected being very low (De Los Santos *et al.*, 2003). This suggests a targeted effect of MB on pathogenic microbes, without interfering with *Trichoderma* functioning.

In general, the success of MB could be related to its high efficacy in suppressing a wide range of pathogenic-microbes causing soil borne diseases (including ARD), over a wide range of soils and environmental conditions (Xie *et al.*, 2015). Furthermore, it is an effective fungicide, herbicide, nematicide and insecticide (Ristaino and Thomas, 1996). The low expense of an effective robust treatment as opposed to numerous treatments with less efficacy is the primary reason why MB has been so successful in the agricultural industry, over such an extended period. However, due to the harsh impact of MB on the environment, implementing alternatives to MB for the control of ARD can no longer be delayed (Yao *et al.*, 2006). A target date of January 2005 and 2015 respectively was set for the complete phasing out of MB by developed (Schneider *et al.*, 2009) and developing countries (López-Aranda *et al.*, 2016; Tripp, 1988) respectively, by the Montreal Protocol or the United Nations Environment Protocol (UNEP).

Overall, the limitations found for current proposed alternative chemical fumigants such as 1,3-D, CP and MS is that they proved to be more sensitive, for optimum efficacy, to soil conditions such as soil moisture and temperature (Desaeger *et al.*, 2017). These alternatives also control weeds, pests and pathogens over a much narrower spectrum, as opposed to using MB (Duniway, 2002). Furthermore, as single treatments 1,3-D and CP provide little management of weeds (Duniway, 2002), but do have potential to be used for the management of nematodes and soil borne fungi (Desaeger *et al.*, 2017). However, when used in combination, these fumigants successfully cover a broad-spectrum of nematodes and most soil-borne fungi including *Pythium*, *Cylindrocarpon*, and binucleate *Rhizoctonia* (Duniway, 2002). Yet, due to the complexity of factors and spectrum of organisms suspected to be responsible for ARD (Yao *et al.*, 2006), only a few of the new alternative chemical fumigants showed good control potential for this syndrome, therefore delaying the progress in managing ARD (van Schoor *et al.*, 2009). The use of 1, 3-D, which has a mode of action through toxicity by alkylation or oxidation, showed effectiveness in reducing bacterial communities such as *Actinobacteria*, but did not completely eliminate them (Zhang *et al.*, 2019). Currently, chemical fumigants such as MS, CP, dazomet, a combination of 1, 3-D and CP, as well as dimethyl disulphide

(DMDS) which could be applied in combination with CP as well as independently of each, are commercially available chemical alternatives to MB for weed control in strawberry cultivation (García-Méndez *et al.*, 2008). Nevertheless, none of the treatments when used alone were considered to have the same efficacy as MB. However, when used in combination better control of weeds was achieved than when applying individual products (López-Aranda *et al.*, 2016).

In a multiphasic approach, the use of steam or chloropicrin as soil treatments in addition to nematicides increased growth and development on apple, pear and cherry seedlings, as opposed to using only nematicides (Mai *et al.*, 1994). Thus, the integration of treatments to effectively irradicate soil-borne pathogens without being harmful to beneficial soil microbes should be investigated in more depth under South-African conditions. This approach promises to provide a long-term solution as well as promote sustainability in commercial monoculture crop production.

ii. Biological alternatives to fumigants

Enhancing soil biological antagonists.

Rumberger *et al*. (2004) proposed to enhance the biological antagonists to ARD by application of compost to the soil. The addition of compost to ARD infected soil as opposed to fumigation has shown to increase soil microbial activity and suppress soilborne pathogens, although no obvious direct benefit with respect to tree performance was found on newly established apple orchards (Yao *et al.*, 2006). The suggested mechanisms of soil pathogen suppression by beneficial microorganisms occurring in the plant rhizosphere is said to be nutrient competition-based due to higher beneficial microbial activity competing for trace elements and nutrients (Compant *et al.*, 2005). An additional consideration is that the possible addition of antagonistic rhizosphere microorganisms to roots as proposed by Sharma *et al*. (2018). This antagonistic reaction could be based upon root exudates harmful to pathogens following infection of the beneficial microorganisms in the plant roots such as antibiotic exudates as well as providing a possible induced resistance response in the crop plants (Line, 2005; Lucas *et al.*, 2018; Sharma *et al.*, 2018).

Van Schoor et al. (2009) reported that compost (sterilized and unsterilized) amendments significantly increased the growth of ‘Golden Delicious’ apple seedlings in a potted trial with ARD infected soil. This was also the case when ARD infused soil was used during field trials, though the growth performance was less significant as in the case of the potted trial. In contrast, Rumberger *et al.* (2004) reported that pre-plant application of compost to apple trees showed no effect on tree growth in an ARD-affected orchard. This lack of response could be ascribed to the effect of the specific site-region combination on the efficacy of treatments, in addition to the specific experiments that were conducted. It is also possible that the origin of the compost (plant and/or animal based) contributed to an introducing of pathogens to the soil and rhizosphere of the trees.

In a study by Van Schoor *et al.* (2009), the use of sterile and non-sterile compost extract amendments were shown to be significantly more successful in increasing the growth of potted apple seedlings planted in ARD soil, compared to the addition of non-biological, chemical treatments such as slow-release fertilizer. This led to the speculation that the mechanism of control by the application of compost extract was not only dependent on the microbial composition of the compost but may also involve the contribution of metabolites extracted from the compost and produced by microbes. Van Schoor *et al.* (2009) proposed that the active metabolites of the sterile compost tea were probably not destroyed during sterilization and were still considered accessible for microbes to metabolize, as in case of non-sterile compost tea amendments. In a later study by Hoitink *et al.* (1997), consistent suppression of disease levels in commercial orchards was reported using compost in addition to biocontrol agents. However, as these results may differ from site to site and are also dependent on the specific etiology of ARD, it should also be compared to potted trials for conclusiveness.

The effectiveness of biostimulants could not be guaranteed under all conditions, since the performance of biostimulants under field conditions are known to be influenced by many factors (Ricci *et al.,* 2019). Broadly defined, biostimulants are substances and/or microorganisms, which may enhance nutrient efficiency, promote tolerance to abiotic stress and improve crop quality when applied to plants (du Jardin, 2015). Seaweed used as a biostimulant on apple seedlings under replant conditions had shown to improve seedling growth by improving soil quality, the change of soil fungal communities through the improvement of soil microbe diversity as well as enhance soil enzyme-activities which in combination leads to an alleviation of ARD (Wang *et al.,* 2016). Similarly, apple seedlings potted in ARD infected soil, treated with different formulations of biostimulants that consisted of a wide range of microorganisms, including mycorrhizal fungi, *Trichoderma* spp., and rhizosphere bacteria (*Bacillus*, *Pseudomonas*, *Rhizobium*) showed improved growth. When these seedlings were subjected to different application rates, an increase in performance was reported with increased application rates (Kelderer *et al.,* 2013). This suggests that the soil microbial populations were altered and led to a positive effect resulting in improved performance of the apple seedlings. In support, study by Thakur *et al.* (2018) in peach orchards showed increased vegetative parameters (plant height, stem diameter and leaf area) that was positively associated with modifications to the replant disease infected soil when using *Trichoderma* and Plant Growth Promoting Rhizobacteria biostimulants, following fumigation.

iii. Alternative approaches

Nutrition

Maintaining an optimum plant nutrition status in the orchards with ARD does not directly promote an increase in growth through the same mechanism as fumigants that address the soil born microbial complex. Yet, nutrition may enhance plant resistance and reduce the negative effect of ARD on plant performance (van Schoor *et al.*, 2009). Of interest is that the growth of apple seedlings in ARD affected soil was increased significantly by adding MAP fertilizer to the soil (van Schoor *et al.*, 2009). This result was concurrent with the findings of Stirk *et al.* (2014) who demonstrated that a healthy plant is more tolerant to pathogenic stresses than a compromised plant.

Rootstocks

One alternative consideration for the control ARD would be to implement ARD-tolerant rootstock. Yet, this field of study has received much less attention than exploring pre-plant chemical fumigants or biostimulants (Rumberger *et al.*, 2004). In a comparison of four apple rootstocks (M793, MM111, M7 and Seedling) planted on ARD infected soil, M793 showed significantly higher plant growth as well as microbial counts (Singh *et al.*, 2017). However, an integration of the various rootstocks with additional treatments such as fumigation and biostimulants produced the best result. Breeding for tolerant rootstocks will lead to a sustainable control measure for replant disease, however, this is time consuming and a better understanding of replant disease and its ethology is needed (Hanschen and Winkelmann, 2020).

Integration with cover crops

Suppressive cover crops such as marigold (*Tagetes patula* cv. Sparky) and wild oats, as alternatives to using pre-plant nematicides and broad-spectrum biocides, were proposed by Mai *et al*. (1994). Marigold in apple orchards were shown as effective as steam pasteurization in suppressing lesion nematodes under orchard conditions and could successfully improve the growth of the apple seedlings planted in the ARD-affected soil. The use of wheat cultivation on ARD infected soils before planting, under greenhouse conditions, also showed promise in controlling some of the pathogens associated with the ARD microbial complex, including that of *Pseudomonas* as well as an introduced inoculate of *Rhizoctonia* and some nematodes (Mazzola *et al.*, 2002). However, the validity of these results appeared to rely on several variables, i.e. the wheat cultivar used, and requires further research.

The use of intercropping alleviated replant disease by adding to the soil microbiology and repelling nematodes (Hanschen and Winkelmann, 2020). Together with management practices such as planting in different tree holes, a reduction of ARD occurrence could take place by limiting the

concentration of apple tree root exudates (phenolics) which have shown to correlate with the occurrence of ARD when in high concentrations (Yin *et al.*, 2016). Ultimately, an integrated method of control would be most effective although prevention of ARD infection in soils should be the first priority (Sharma *et al.*, 2020).

6. CONCLUSION

Replanting of new orchards on previously cultivated land will only increase in future in commercial apple production systems, as globally virgin soils are decreasing in the traditional fruit growing areas. However, replanting existing orchards with a rotation crop to apples to reduce the impact of ARD is not always possible, therefore fumigation is as a rule required to maintain a profitable income. Although MB has been very effective in controlling the negative effect of ARD in apple trees, there were incidences of negative results on both performance and beneficial soil microbial populations. Since 2015, this product has been totally banned for the use of fumigation of fruit crops, therefore necessitating the urgent search for alternative solutions to address ARD in apple. Yet, the current use of alternative chemical fumigants or combination fumigation strategies face serious challenges regarding varying performance success that is highly reliant on specific environmental and soil conditions. In addition, the pressure from retailers and consumers alike to farm and produce sustainably will prioritize the selection of environmentally friendly alternatives as an option to combat ARD within the commercial horticultural sector in future.

Existing alternatives indicate selective efficiency against specific organisms in the ARD complex and do not currently provide the same broad-based control of MB. Thus, a single treatment or product to address ARD may not be sufficient to achieve comparative high yields to that possible under the previous scenario when MB was the fumigant of choice. However, a combination of alternatives either through indirect alleviation of stress, by increasing resilience of the plant, and/ or by increasing the microbial populations, may be required to control ARD. More research is required to investigate alternative approaches and explore new products for the effective control of ARD that is not only for greater efficacy, but also to be more environmentally sustainable within a commercial production context.

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PAPER 1: The Effect of Biostimulants on the Root Growth Dynamics of Young Apple Trees Established in Fumigated Soil

ABSTRACT

Soil fumigation, an essential practice in the establishment of orchards on soils where similar crops were previously cultivated, assists to overcome the inevitable orchard replant disease and obtain optimal fruit quality and yield. Biostimulants were considered for remediation of the biological soil microbiome and reduction of abiotic stress conditions after chemical soil fumigation to enhance root growth. The effect of limited applications of five commercial biostimulants on the root growth of young 'Granny Smith/MM109' trees after fumigation were evaluated during 2016/17, and evaluation continued for three treatments during 2017/18 by means of destructive and non-destructive techniques (minirhizotron). Treatments comprised an untreated Control, Compost, Plant Extract, L-Amino Acids (LAA) and two Trichoderma products (Trich 1 and 2). Seasonal trends during 2016/17 separated treatment effects into three distinct groups regarding root dynamics: Mycorrhizae; Trich 1 and Trich 2; Other. Significant differences between treatments varied per evaluation date and soil depth. Seasonal total root number (TRN) and root length (TRL) trends showed consistent low root growth in the Mycorrhizae treatment, followed by the untreated Control, Compost, Plant Extract and LAA treatments, with the highest values in the Trich 1 and 2 treatments. Seasonal trends in total root area (TRA) and total root volume (TRV) were the complete opposite and the Mycorrhizae treatment had the highest root volume and area. Initially TRA and TRV were higher in the Trich 1 and 2 treatments than the other treatments. During 2017/18, significant differences between the three treatments were only obtained in the topsoil layers (0-20 cm). The Control differed significantly from the Compost and Plant Extract treatments and confirmed an effect of biostimulants on root dynamics after two seasons. Significant differences between treatments varied according to root diameter and soil depth in the destructive study in 2017/18. Overall, our results provided evidence that root dynamics were influenced by limited applications of biostimulants at establishment of trees after fumigation. Efficacy differed according to the mode of action of the biostimulant and could probably increase with increasing the frequency of application.

Key words: *Apple Replant Disease, Chloropicrin, 'Granny Smith', Microorganisms, Minirhizotron*

1. INTRODUCTION

In the Western Cape, South Africa, virgin soil for agricultural expansion is limited. Therefore, farmers are often without choice but to establish new apple orchards on soils previously planted with pome fruit. This practice implies major challenges such as facing potential soil borne disease pressures that are known to negatively impacts newly established orchards. One such common soil pathogen complex (Tewoldemedhin *et al.,* 2011) that occurs on replant sites is known as apple replant disease (ARD). This condition results in major losses in apple production by negatively affecting the fruit crop at various levels throughout the phenology, including that of tree establishment, growth, yield and fruit quality (Van Schoor *et al.,* 2008). In addition, the impairment of the tree root system caused by the damage with transplanting from the nursery to the orchard, also often results in stunting of growth (Leinfelder and Merwin, 2006; van Schoor *et al.,* 2009).

Lucas *et al*. (2018) reported apple roots affected by ARD to show symptoms of necrosis, reduced root growth and lower root hair numbers. Close contact of crop roots with ARD-infected soil not only compromises their growth and morphology, but also reduce the ability of such root systems to acquire specific nutrients including, but not limited to, nitrogen (Lucas *et al.,* 2018), molybdenum and iron (Fazio *et al.,* 2012). This is especially applicable within the first year following transplanting, a period in the tree phenology that is crucial for tree canopy development. In addition, soil pH and soil type too influences nutrient uptake, therefore nutrient deficiencies should be interpreted along with the site-specific conditions for each orchard and cannot be ascribed to the effect of ARD in isolation (Fazio *et al.,* 2012).

White root tip dynamics of young, non-bearing 'Royal Gala' trees monitored in the Vyeboom region, Western Cape, indicated active white root growth during the entire season (Van Zyl, 2016). In this study, 'Royal Gala' trees on M7 root stock were established in a sandy loam soil after the soil was fumigated and treated according to standard commercial amelioration practices. The aim of the study was to increase soil microbial life for improved soil conditions, by establishing different cover crops in the work rows. However, no direct stimulation of root growth was noted, with any of the cover crop treatments. A limitation of this study was that information on the general root growth status such as the number of root hairs were not reported.

Pre-plant control methods such as soil fumigation has been a well-established commercial practice to combat the effect of ARD in apple orchards and control the factors known to contribute to poor establishment and plant performance (Lucas *et al.,* 2018; Tahir, 2006). However, the most effective soil fumigant, methyl bromide (MB), has been discontinued globally, due to its harsh impact on soil biota and possible carcinogenic effect on human health (Lazarovits, 2001). Although viable alternatives to MB such as 1,3-Dichloropropene (1,3-D) and chloropicrin (CP) have been identified

(Noling and Becker, 1994), these compounds have not been able to provide complete control of broad spectrum pathogenic complexes, as well as weeds when applied as single treatments (Duniway, 2002; García-Méndez *et al.,* 2008). Nevertheless, when used in combination the fumigants 1,3-D and dimethyl disulphide were successful in the suppression, but not the complete elimination, of soil pathogens such as *Phytophthora* spp. and *Fusarium oxysporum,* compared to the untreated control plots (Mao *et al.*, 2016).

One approach to remediate the compromised natural microorganism balance in fumigated soil is using biostimulants (Sahain *et al.*, 2007). Biostimulants are defined as any substances and/or micro-organisms applied to the rhizosphere to enhance nutrient uptake and efficiency by promoting, amongst others, soil microbe content, with the aim to increase tolerance to abiotic stress as well as promote crop quality (Du Jardin, 2015; Egamberdieva *et al.,* 2017; Rouphael *et al.,* 2020). One such biostimulant, the plant growth promoting rhizobacteria (PGPR) *Bacillus,* has been shown to effectively increase lateral root growth and the number of root hairs, while inhibiting primary root growth in soybean (Bavaresco *et al.*, 2020). Along with increased root length, significant alterations to root architecture include increases in total diameter, volume and root surface following the inoculation of plant growth promoting bacteria (PGPB), such as *Trichoderma* spp. and mycorrhiza (Verbon and Liberman, 2016). However, as beneficial microorganism communities around plant roots are highly influenced by soil type (Nallanchakravarthula *et al.,* 2014), soil moisture (Dijkstra and Cheng, 2007), soil temperature and the particular stage of plant phenology (Moyano *et al.,* 2007), results may vary between orchards and production sites and regions (Nallanchakravarthula *et al.,* 2014).

An unintentional effect of the pre-plant application of 1,3-D and chloropicrin (CP), aiming to control soil-borne diseases such as nematodes and parasitic fungi, is that non-targeted soil microbes are most often terminated along with the pathogenic soil biota (Pecina *et al.,* 2016). Alternatively, beneficial bacteria in the rhizosphere may be compromised due to enhanced competition with pathogenic bacteria (Yang *et al.,* 2001) that were not completely eradicated by fumigation (Klose *et al.,* 2007). Both these outcomes are likely to alter the favourable rhizosphere conditions required by the young apple tree roots for optimal growth (Morgan *et al.,* 2005). The eliminating effect of fumigation on beneficial soil microbes may be limited to the robust survival of wild natural occurring species. In their study, Naseby *et al*. (2000) showed that *Trichoderma* spp. survived being autoclaved, whereafter it still had a promotive effect on the growth of young seedlings resulting in increased shoot and root weight.

The aim of this study was to evaluate the efficacy of a range of commercial biostimulant products, with different modes of action, on the root growth dynamics of young ‘Granny Smith’ apple trees, after transplanting in a fumigated soil. The objectives were firstly, to evaluate and quantify the

effect of the various biostimulant treatments on apple tree root growth during the first season, using non-destructive, *in-situ* minirhizotron images. A second objective was to further evaluate the effect of three selected treatments on the root distribution of young apple trees in the soil profile after two seasons, but now also following a two-dimensional, destructive root analysis approach, in addition to *in-situ* images. Results from this study aim to inform on the efficacy of specific biostimulants to significantly increase root growth after fumigation, and to identify suitable treatments that can be employed to reduce the effect of transplant shock on establishing new apple orchards, specifically on potential ARD soils.

2. MATERIALS AND METHODS

Experimental site

The experimental site was located on a commercial farm, Lovenstein, in Vyeboom (34°04'55''S; 19°04'12''E), South Africa. The experiment was conducted on 'Granny Smith'/MM109, planted on 22 October 2016, at a planting distance of 5 m x 2 m (Fig. 1). Soil preparation prior to planting was done by the producer following standard procedures and included deep excavation and adjustment of chemical mineral balances. Plant rows were ridged (47 cm x 213 cm) to compensate for soil depth. The soil profile of the chosen orchard was classified as a combination of a medium sandy Katspruit loam and a sandy Longlands loam (Macvicar *et al.,* 1991) for which ridging of approximately 50 cm x 200 cm is recommended in the planting row. Soils were supplemented pre-planting with the nutrient-based ameliorants Maxi Phos (Omnia Nutriology®) and KCl (Yara Africa Fertilizer Pty, Ltd., Paarl) on 20 May 2016 at 500 and 300 kg. ha^{-1}, respectively. 'Enhancer' consisting of pelletized chicken manure (InteliChem Pty Ltd., Wellington) at 1.2 $kg.tree^{-1}$ was added at planting to enhance water holding capacity, improve soil organic content and stimulate microbial activity. 'Aldo' (unknown source), a controlled release NPK fertilizer, was applied two months after planting, at 200 $g.tree^{-1}$ in December 2016 and again, 13 months after planting, in November 2017. Compost-tea (Ecosoil, Grabouw) was applied at a rate of 240 $L.ha^{-1}$ (approximately 1000 trees) at monthly intervals from November 2016 to February 2017.

The micro-irrigation was installed 5 days after planting, and during the period prior to the installation of the irrigation system irrigation of trees was conducted manually at approximately 15-25 litres per tree per day. The micro-irrigation schedule for the 'Granny Smith' trees was applied for eight hours, once a week, for the first month after planting. For the 2016/17 season, from December to February and again from May to November 2017 the trees were irrigated for five hours, every 6^{th} day. During March and April, trees were irrigated for an additional hour, with every second cycle. For the 2017/18 season, the trees were irrigated every 5^{th} day for four hours and again for an additional

two hours, during March and April, with every second cycle. Irrigation required was adjusted from 2016 to 2018, due to a severe drought that was experienced (Figs. 2-4).

Fumigation, using a combination of 1,3-D (490 $g.L^{-1}$) and CP (710 $g.L^{-1}$), was performed on ridges only, during September 2016, approximately seven weeks prior to planting, by a commercial company (BioScience Research, Cape Town, 7550), according to standard procedures.

Treatments

Six treatments were applied at planting, according to a randomized complete block design (RCBD), on 22 October 2016, strictly following protocols as specified on each product recommendations to ensure optimum efficacy of each treatment (Table 1). An untreated control was included and managed according to the same standard orchard management practices as treated units. Ten trees per treatment and untreated control were used, where single trees served as experimental units. One buffer tree was included between treatments and treatment rows were separated with a buffer row.

Data collection

Root Scans

At planting, 21 trees (three replicate trees for each of the six treatments and untreated control) were selected according to a completely randomized design, for root studies. A clear acrylic tube, of 100 cm x 7 cm, sealed at the bottom end and covered at the top, was installed at a 45 ° angle in relation to the soil surface, approximately 10 cm from the tree trunk, perpendicular to the tree row (Fig. 5). From December 2016 and onwards, measurements commenced monthly, using a minirhizotron root scanner (CI-600 In-Situ Root Imager, CID Bio-Science INC.). Four images per tube, one at each of four soil depth intervals (45 – 60 cm, 30 – 45 cm, 15 – 30 cm and 0 – 15 cm), were taken.

During the first season (15 Dec 2016 to 02 Oct 2017), all six treatments and the untreated control were monitored, whereas during the second season (28 Nov 2017 to 31 Aug 2018), only three treatments, namely the Control, Compost and Plant Extract treatments, were selected for continuous monitoring. The images were analysed, using the Root Snap Image Analysis Software (CI-690, Version 1.3.2.25, CID Bio-Science INC.) (Fig. 6). Data were exported to XML format for statistical analysis. Individual root data per image were recorded by counting root numbers and measuring root length and diameter (mm), from which root volume and area was calculated. Roots maintained their identity until becoming non-functional (dead/black) or were no longer visible for imaging next to the minirhizotron. Identification of root activity and colour during the different developmental stages was fully described by Cameron (2019) and was used to identify dead and active roots. Root data per image represented an area of 19.6 cm x 19.6 cm, thus approximately 384 cm^2.

Destructive Root Analysis

Destructive root analyses were performed (n=3) approximately two years after planting, on 17 July 2018, for the untreated Control, Compost and Plant Extract treatments, according to the methodology of Böhm (1979) and Van Zyl (2016). Visible roots within a 100 cm x 100 cm area were counted (Fig. 7) and classified according to diameter, using an electronic calliper (150 mm LCD Digital Electronic Carbon Fibre Vernier Calliper Gauge Micrometre Measuring Tool).

Soil moisture and temperature

A DFM continuous logging soil moisture probe (DFM Technologies PTY LTD, Pniel) was installed in close proximity of a representative tree to record soil moisture (Figs. 8-10) and soil temperature (Figs. 11-13) at six depths in the soil profile (10 – 40 cm; 60 – 80 cm at 10 cm intervals respectively). The data was collected during a period from October 2016 to December 2017.

Statistical analysis

Statistical analyses were performed using One-way Analysis of Variance (ANOVA), according to the generalized linear model (GLM) procedure in SAS 9.4 (SAS Institute Inc. 2004, Cary, USA). Means were separated by means of Fishers' posthoc Least Significant Differences (LSD) test, where significant differences were considered at a 5% confidence level ($p \leq 0.05$). In addition, regression analyses were performed, per tube, representing seasonal trends of the various treatments over time. This was presented with confidence levels and SE values using XLSTAT (XLSTAT statistical and data analysis solution. New York, USA. https://www.xlstat.com)

3. RESULTS

Root Images
2016/17 SEASON

Total root number (TRN)

Level 1(45-60cm)

Significant differences for TRN were found in level 1 (45-60 cm soil depth), only for Oct 2017, where the trees that received the mycorrhizae treatment had significantly lower TRN than trees treated with any of the other treatments, including the untreated control (Table 2). Trees treated with Plant extract had a significantly higher TRN than recorded for the Compost treatment, but did not differ significantly from LAA, Trich 2 and the Control (Table 2).

Level 2(30-45cm)

TRN reported at level 2 (30-45 cm soil depth) showed significant differences between treatments from Jan – Mar 2017, in July and again in October 2017 (Table 2). TRN counted on trees that were treated with Mycorrhiza was consistently low but did not always differ significantly from other treatments throughout the season. In Jan 2017, the Mycorrhiza treatment showed a significantly lower TRN from the Control, as well as the Trich 1 and Trich 2 treatments, but not from Compost, Plant Extract and LAA. For both February and March 2017, the Mycorrhiza treatment displayed significantly less TRN than all other treatments, apart from the LAA treatment in February (Table 2). No significant differences between any other treatments (Plant Extract, Trich 1, Trich 2 and Compost) and the Control were observed during February and March 2017, with all other treatments showing a significantly higher TRN than trees treated with Mycorrhiza and LAA. Significantly fewer roots were produced during July and Oct 2017 for Mycorrhizae compared to the TRN of the Control Plant Extract treatment. No significant differences in TRN were found between the Control and Compost, Plant Extract, LAA, Trich 1 and Trich 2 treatments during July and Oct 2017 (Table 2).

Level 3 (15-30cm)

Significant differences in TRN were recorded throughout the season in level 3 that represented a soil depth of 15-30 cm (Table 2). In Dec 2016, trees from the Trich 1 treatment displayed significantly higher TRN compared to all other treatments and the untreated control, which did not differ from each other. In Jan 2017, TRN for trees that received Trich 1 was significantly higher than for trees treated with Mycorrhizae, LAA and Compost, but were not significantly different from the Control, Plant Extract and Trich 2 (Table 2). In Feb 2017, TRN noted on trees from the Mycorrhizae treatment were significantly lower than the Control, Plant Extract, as well as the Trich 1 and Trich 2 treatments, but did not differ significantly from the Compost, and LAA treatments. None of the other treatments differed from each other or the Control. Mar 2017 showed significantly higher TRN for Trich 1-treated trees compared to the TRN that was reported in trees that received LAA, Mycorrhizae and Compost, but did not differ significantly from the Control, Plant Extract and Trich 2 treatments, which also did not differ from the Control (Table 2). In Apr- and May 2017, the TRN of trees treated with Mycorrhizae and LAA were significantly lower than all treatments except that of the Composs treatment, while the other treatments (Plant Extract, Trich 1 and Trich 2) did not differ significantly from one another or the Control (Table 2). In Jul 2017, Mycorrhizae had significantly lower TRN than the Control, Plant Extract and Trich 1 treatments, LAA was significantly lower than the Control but did not differ from the Trich 1 and Trich 2 treatments. Both Trich 1 and Trich 2 treatments did not differ significantly from the Control of Plant Extract. In Aug 2017, the highest TRN were recorded

in the Control and Plant Extract treatments, which differed significantly from the Compost, Mycorrhiza and LAA treatments, but not from Trich 1 and Trich 2. In Oct 2017, Plant Extract showed a significantly higher TRN than Compost, Mycorrhiza and LAA (which did not differ significantly from each other), although it did not differ significantly from the Control and Trich 2.

Level 4 (0-15cm)

In the topsoil at level 4 (0 – 15 cm), significant differences between treatments were noted in Dec 2016 and Jan 2017, as well as from Apr- to Oct 2017 (Table 2). In Dec 2016, trees treated with Compost, Mycorrhiza and LAA had a significantly lower TRN than those treated with Trich 1 and the Control (Table 2) but did not differ significantly from the TRN recorded in trees that received the Plant Extract or Trich 2 treatment. In Jan 2017, the TRN for treatments Compost, Mycorrhiza, Plant Extract and LAA were significantly lower than the Control, which did not differ from Trich 1 and Trich 2. In April 2017, the Compost, Mycorrhiza, Plant Extract and LAA treatments displayed had a significantly lower TRN than the Trich 2 and Trich 1 treatments, although no treatments differed significantly from the Control. In May 2017, the Compost, Mycorrhiza, Plant Extract and LAA treated trees displayed had a significantly lower TRN than the Trich 2 and Trich 1 treatments as well as the Control. In July 2017, the Control showed a significantly higher TRN than Compost, Mycorrhiza, Plant Extract and LAA. No significant differences between Trich 1, Trich 2 and the Control were observed during July 2017, although Trich 1 and Trich 2 showed a significantly higher TRN than LAA and Compost. For the Oct 2017 period, Mycorrhizae had significantly lower TRN than that recorded for the Control and Plant Extract treatments, but did not differ significantly from the remaining treatments (Table 2).

Mycorrhiza generally had the lowest TRN of all treatments, irrespective of soil depth, and remained constant throughout the season (Table 2). The TRN of Trich 1 and Trich 2 showed varying trends during the season, but a general increase in TRN was noted from Dec 2016 until Jul 2017, where after numbers declined, until a slow increase towards Oct 2017 was noted, which was similar for all depths. TRN in Trich 1 and Trich 2 was noticeably higher than for Mycorrhizae, but comparable to that of other treatments. Increasing trends in TRN from Dec 2016 towards Oct 2017 were noticed in the Control and Plant Extract treatments, for all depths, with a similar but less pronounced trend that was noticed for the Compost and LAA treatments. Both LAA and Compost treatments showed an initial increase in TRN. A sharp decrease in TRN that was noticed in treatments Trich 1 and Trich 2 for the Jul 2017 observation period.

Total root length (TRL)

Level 1(45-60cm)

When considering TRL for the trees observed from Dec 2016–Aug 2017 at 45-60 cm soil depth (level 1), no significant difference was reported between treatments (Table 3). However, in Oct 2017, Mycorrhizae had a significantly lower TRL than Plant Extract, although not significantly so from any other treatment (Compost, LAA and Trich 2) or the Control. A similar trend was also noted from Jan 2017 to Aug 2017, where the Mycorrhizae treatment displayed an overall lower TRL compared to other treatments, not significant at the 5% confidence level.

Level 2(30-45cm)

For the soil depth 30-45 cm, significant differences for TRL were found between treatments for Feb – Apr 2017, Jul- and Oct 2017. Again, a similar trend was noticed as in the lower soil depth of 45-60 cm, where the Mycorrhizae treatment consistently recorded lower TRL in 2017, although not always significantly different from the other treatments (Table 3). In Feb 2017, trees treated with Mycorrhizae had a significantly lower TRL compared to Compost, Plant Extract, LAA, Trich 1 and Trich 2 treatments, except the untreated Control. In turn, the Control had a significantly lower TRL than the Trich 1 and Trich 2 treatments, but did not differ significantly from the remaining treatments (Compost, Mycorrhiza, Plant Extract and LAA) (Table 3). In Mar- and Apr 2017, the Mycorrhizae treatment had a significantly lower TRL than all other treatments, whilst these treatments did not differ from one another, except in Apr 2017 where the TRL of the Mycorrhizae treatment was comparable to that of the Control. In Jul 2017, the Plant Extract treatment had a significantly higher TRL than the Compost, Mycorrhizae, and LAA treatments, but did not differ significantly from the Control, Trich 1 and Trich 2 treatments. A similar result as seen in Jul 2017 was obtained for the Oct 2017 period, except that no results for Trich 1 could be collected (Table 3).

Level 3 (15-30cm)

Roots located at 15-30 cm soil depth (level 3) displayed significant differences in TRL between treatments throughout the season (Table 3). The Mycorrhizae treatment consistently recoreded the lowest TRL but did not differ from the Control in Dec 2016 and Jan 2017 (Table 3). In Dec 2016, the Trich 1 treatment displayed significantly higher TRL compared to all treatments and the Control. For this period, LAA treatments showed a significantly lower TRL than recorded for both Trich 1 and Trich 2 treatments, although it was comparable to that of the remaining treatments (Compost, Plant Extract, Mycorrhiza) and the Control. In Jan 2017, Trich 1displayed a significantly higher TRL than LAA, Mycorrhizae and Compost, but did not differ significantly from the Control or Plant

Extract and Trich 2. For the same period, Mycorrhiza showed the lowest TRL, although it did not differ significantly from the Control, Compost and LAA treatments (Table 3). For Feb 2017, the lowest TRL was observed in the Mycorrhizae treatment, but numbers were comparable to that of the Compost and LAA treatments, whilst Trich 1, Trich 2, Plant Extract, Compost and the Control were significantly higher for this period (Table 3). No significant differences were recorded between the last-mentioned treatments and the Control. In Mar 2017, TRL of the Plant Extract, Trich 1 and Trich 2 treatments were significantly higher than the LAA, Mycorrhizae and Compost treatments, but did not differ significantly from the Control. During this period, Mycorrhiza had a significantly lower TRL than the Control, but did not differ significantly from Compost and LAA. A similar trend as in Mar 2017 was noticed in Apr 2017, except that TRL of the LAA treatment was significantly lower than recorded for the Control (Table 3). In May 2017, Trich 2 had a significantly higher TRL than the LAA, Mycorrhizae, Compost treatments and the Control, but were comparable to that of the Trich 1 and Plant Extract treatments. The LAA and Mycorrhiza had a significantly lower TRL compared to the Control during this period. No significant differences between the Control and Trich 1, Plant Extract and Compost treatments were observed during May 2017. For the Jul 2017 period, Plant Extract had a significantly higher TRL than Compost, Mycorrhiza, LAA and Trich 2, but not the Control, Trich 1 and Trich 2 treatments, which also did not differ from one another. In Aug 2017, Plant Extract had a significantly higher TRL than LAA, Trich 2, Mycorrhizae and Compost, but were comparable to the Control and Trich 1 treatments. Mycorrhiza had a significantly lower TRL than the Control and Plant Extract during the same period. In Oct 2017, the Plant Extract treatment had a significantly higher TRL than all other treatments, except for the Control, which was similar. Significantly lower TRL values were recorded for the Mycorrhizae treatment compared to the Control and Trich 2 treatment. The TRL of the Control did not differ significantly from the Compost, Plant Extract and Trich 2 treatments (Table 3).

Level 4 (0-15cm)

For the topsoil layer with a depth of 0–15 cm (Level 4), significant differences between TRL values were recorded for all dates, except for Dec 2016 and Feb 2017 (Table 3). In Jan 2017 LAA had a significantly lower TRL compared to that of the Trich 1 and Trich 2 treatments and the Control, but did not differ significantly from the Compost, Mycorrhiza and Plant Extract treatments. In Mar 2017, Trich 1 and Trich 2 had a significantly higher TRL than Mycorrhiza, Plant Extract and LAA, although no significant differences between any treatments were seen when compared to the Control (Tabel 3). During April 2017, Trich 1 and Trich 2 had a significantly higher TRL compared to Compost, Plant Extract and LAA. However, none of the treatments differed significantly from the Control. In May 2017, Trich 1 and Trich 2 had a significantly higher TRL than Compost, Mycorrhiza, Plant

Extract and LAA, but did not differ significantly from the Control. During this period Mycorrhiza, Plant Extract and LAA had a significant lower TRL than the Control. In Jul 2017, Compost, Plant Extract and LAA had a significant lower TRL than Trich 1 and the Control. The Control showed a significantly higher TRL compared to all treatments except for Trich 1. In Aug 2017, TRL of Compost and LAA was significantly lower than the Control and Trich 1, but did not differ significantly from Mycorrhiza, Plant Extract and Trich 2. For the Oct 2017 period, the TRL for Mycorrhiza an LAA was significantly lower than that of the Control and Trich 2, but did not differ significantly from Compost and Plant Extract. The Control showed a significantly higher TRL than Compost, Mycorrhiza and LAA, but did not differ significantly from Trich 2 and Plant Extract (Table 3).

Seasonal trends for TRL separated the treatments into three groups (Fig 21). The Mycorrhizae treatment generally showed the lowest initial (from Jan 2017 – May 2017) TRL, with a minor increase towards the end of the season in July 2017 and a then a slight decrease towards Oct 2017. A higher initial TRL was noted in the Control, Compost, Plant Extract and LAA treatments, whereas Trich 1 and Trich 2 generally displayed the highest TRL throughout the season, with the exception of Jul 2017 to Oct 2017, where the Control and Plant Extract treatment displayed a higher TRL. However, in contrast to trends noted for TRN, a predominant linear increase in TRL throughout the season was observed for the rest of the treatments, displaying a short decline from May to Jul 2017, before it increased towards Oct 2017. For the deeper soil depths of levels 1 and 2, TRL trends ranked as follows: Control, LAA, Compost and Plant Extract treatments in an increasing order, with the highest TRL observed in the Plant Extract treatment.

Total root area (TRA)

For TRA, no significant differences were recorded between treatments for the lower soil levels 1 and 2 (Table 4). For the soil depth 15-30 cm, a significant difference in TRA was only observed in Jan 2017, where Mycorrhizae had significantly more TRA compared to any of the treatments. When considering the topsoil layer of 0-15 cm in Dec 2017, Mycorrhizae showed a significantly higher TRA compared to all treatments, except Trich 2. However, TRA for the Trich 2 treatment did not differ significantly from the remaining treatments. During Jan 2017, Mycorrhizae had a significantly higher TRA compared to all other treatments. No significant differences were observed for this level for the rest of the season (Table 4).

Total root volume (TRV)

When considering TRV, Mycorrhizae consistently recorded the highest values throughout the season, in all four soil depths, although not always significantly so (Table 5). Significantly higher TRV's was observed in the Mycorrhizae treatment for Jan, Feb, Aug and Oct 2017 for soil level 1, whereas

for soil level 2, Jan, Mar, Apr, May and Oct 2017 recorded significantly higher TRV values. For the level 3 soil depth, the Mycorrhizae treatment displayed significantly higher TRV during Jan, Feb, May, Aug and Oct 2017, whereas Dec 2016 and Mar 2017 recorded significantly higher TRV compared to all other treatments (Table 5).

Seasonal trends for TRV are illustrated in Fig 22 and 23. The TRV for Mycorrhiza was substantially higher than the other treatments (Fig 22), and was thus illustrated separatedly (Fig 23). The Mycorrhiza treatment displayed a high initial TRV in Jan 2017 after which it declined towards Feb 2017 and increased again to Mar 2017 (Fig 23). This trend was seen in the other treatments and the Control. In contrast, the Mycorrhiza TRV steadily declined from Mar 2017 towards May 2017, whereas all other treatments and the Control steadily increased. In July 2017, all treatments displayed a decline in TRV, with the exception of the Mycorrhiza treatment, which displayed a drastic increase. In Aug 2017, all treatments and the Control, showed an increase, whereas Mycorrhiza showed a decrease, in TRV. In the period Aug 2017 to Oct 2017, Mycorrhiza displayed a sharp increase in TRV compared to all other treatments and the Control (Figs 22 and 23).

2017/18 SEASON

Total root number (TRN)

For the 2017/18 season, significant differences in TRN were only detected in the topsoil layer (0 – 15 cm). The Control consistently outperformed the Plant Extract and Compost treatments with regard to TRN (Table 6) for the period of Jan – Feb 2018 and again from May – Aug 2018. TRN was significantly higher in the Control for Jan, Feb, May, Jun and Aug 2018 (Table 6).

Total root length (TRL)

No significant differences between treatments were found for TRL in the deeper soil levels 1 or 2, throughout the season (Table 7). However, at soil level 3, the Control showed a significantly higher TRL than the Compost and Plant Extract treatments for Jan 2018, Apr 2018 and both Aug 2018 dates, except for Apr 2018 when no data for Compost could be collected. A similar trend continued for the topsoil depth of 0-15 cm (level 4) for Jan, Feb, Apr, Jun, and both Aug dates during 2018 (Table 7). For Jan 2018, there was a significantly higher TRL in the Control compared to the Compost and Plant Extract treatments. For Jan 2018, Plant Extract showed significantly lower TRL compared to Compost (Table 7).

Total root area (TRA)

No significant differences were noted for TRA between the Control and other treatments, for the deeper soil levels 1 and 2, throughout the season (Table 8). The first significant differences were

noted towards the end of the season, at a level 3 soil depth (15-30 cm), for both Aug dates in 2018 , when the Control displayed a significantly higher TRA than the other treatments. This trend was repeated for the topsoil for Jan, Feb and Apr 2018. However, no further significant differences between treatments or the Control were recorded for the rest of the season (Table 8).

When considering general trends over the two seasons (Annexure A), TRA values for Mycorrhizae were not only substantially higher initially, but also consistently hither throughout the first season, for all soil depths. Although a mostly increasing linear trend was noted, this trend consisted of several fluctuations during the first season. Trich 1 and Trich 2 treatments showed the second highest TRA values, similar to that observed for the TRN and TRL values observed during the first season, although the intensity varied between treatments and soil depths. These values peaked around Apr 2017 and May 2017, before declining sharply towards Jul 2017. Thereafter, TRA values showed an increasing trend, but final values were lower that those in the initial autumn peak. The highest TRA values were observed in the Control, at the topsoil level, during the second season, with only three treatments remaining. However, with increasing soil depth, TRA for the Compost treatment displayed the highest values between the respective treatments for that soil depth (Annexure A).

Total root volume (TRV)

Significant differences for TRV for the deepest soil level (45-60 cm) was only observed on 2 Aug 2018, where the Compost treatment showed a significantly higher TRV than the Plant Extract treatment, although both these values were comparable to the Control (Table 9). At level 2, significant differences were recorded in Nov 2017, May and Jun 2018, where Compost had a significantly higher TRV compared to the Control and Plant Extract treatments. During 2 and 31 Aug 2018, Compost had a significantly higher TRV than the Plant Extract treatment, although neither differed significantly from Control (Table 9). At soil depth level 3, no significant differences during the season were detected between treatments (Table 9). For the topsoil (0-15 cm), significant differences were only observed for in Feb and Apr 2018, when the Control displayed a significantly higher TRV than the Plant Extract and Compost treatments, which did not differ significantly from each other. Seasonal trends in TRV showed three distinct treatment groups displaying similar seasonal dynamics described above for TRA (Annexure A).

Destructive Root Analysis

Most of the roots were in the < 2mm class and thus root numbers were analysed as total root numbers in Table 10. No significant differences between treatments were found for total root number, throughout the profile (Table 10). Root distribution in the soil profile is illustrated in Figs. 14–16 for

the different soil depths. The distribution of the few roots with diameter < 2 mm showed no clear trend. Treatment differences are shown in Figs. 17-20. Significant differences between treatments varied according to soil depth. Although significant differences between treatments for diameters < 2 mm occurred at different soil depths, it varied, and no clear trend was visible (Fig. 17). The number of visible roots in the top soil layers (20-30 cm) were higher in the Control, Compost and Plant Extract treatments compared to the deeper soil layer (20-40 cm), with little to no roots recoreded at a depth of 90 – 100 cm (Table 10). Fig. 18 showed treatment differences for root diameters 2–5 mm. These roots were not present in all treatments at all depths and were only recoreded at 20 cm soil depth,where a significantly higher root number was found in the Control compared to the Compost and Plant Extract treatments. Similarly, roots with diameters 5-10 mm did not occur in all treatments at all depths (Fig. 19). Significantly more roots occurred in the Compost compared to other treatments at 30 cm soil depth. Finally, roots with diameter > 10 mm only occurred in the Compost treatment, therefore no significant results are presented.

Soil probe data

DFM data is presented as a series of figures that show the soil moisture (Figs. 8-10) and soil temperature (Figs. 11-13) for the different depths in the orchard. Soil moisture data from the DFM probes indicated severe stress in the 30–80 cm soil depths during the first two months after planting (Figs. 8-9). However, thereafter soil moisture recovered with the installation of the micro-irrigation system. Soil temperatures did not fluctuate dramatically between soil depths (Figs. 11-13) and is comparable to earlier data recorded for the Western Cape (Lötze, 2012). The development of roots beyond 80 cm, as reflected in the destructive root study, serves as an indication that sufficient soil moisture for normal root development was available. However, the low soil moisture in the top 30 cm soil layer during Oct and Nov 2016 may have compromised the performance of the applied treatments (Fig. 8).

4. DISCUSSION

2016/17 season

During the 2016/17 season, no treatment consistently and significantly outperformed another, for all soil depths. Strong indications of treatment dominance, specifically Plant Extract, Trich 1 and 2 and the Cntrol in the topsoil depths often disappeared in lower soil depths. This may partly be due to known variation between trees in field trials, or where the rhizosphere of a rootstock soil depth increased outside the physical range of possible biostimulant interaction. Such varying response of biostimulants to different soil textures and soil moisture have been reported before (Moyano *et al.*, 2007; Nallanchakravarthula *et al.*, 2014). The soil texture in our experimental site was compromised

after soil preparation and ridging, which could have contributed towards the varied responses of the treatments. In addition, unfavourable soil moisture conditions experienced during the early establishment phase of the trees could have contributed to the variation in the efficacy of the respective biostimulants. In addition, a more comprehensive network of soil probes is required to better quantify environmental effects on biostimulant performance.

Root growth, together with soil fertility, are important factors to consider when dealing with abiotic and biotic stress after planting a newly established orchard in fumigated soil. It is evident from TRN results that roots were present throughout the year, albeit through new growth or remaining throughout the seasons measured as described in the materials and method, similar to previous findings under South African conditions that indicated continuous root growth in young apple trees throughout the season (Lötze *et al.*, 2018; Van Zyl, 2016). However, the purpose of the study was to capture all root growth, throughout the study, including active, young root growth to capture the expansion of the root system. Root numbers varied between treatments, soil depth and evaluation dates. It is important to note that only the visible roots were counted and measured, therefore if the visibility of some roots was obscured by soil surrounding the PVC tube over time, these roots would not be visible and therefore not be counted. TRN was generally the lowest in the Mycorrhizae treatment in the 2016/2017 season (approx. 20), for all soil depths, although it was not always significantly so in the topsoil layer (0-15cm) during the month of July. This lack of consistent differences may be partly due to the high variation between tree replicates under field conditions, also reported by Van Zyl (2016).

Initial (Dec. 2016) TRN values for LAA and Compost treatments were higher (20-40 TRN) than that recorded for the Mycorrhizae treatment (Table 2), but lower than the other treatments as the highest initial TRN were reported for the Trich 1 and Trich 2 treatments (40-80 TRN) (Table 2). Clear seasonal trends noted between treatments for TRN can possibly be ascribed to the different mode-of-actions used by the diverse biostimulant categories represented in this trial (Rouphael *et al.*, 2020). However, it was not possible to show significant differences in TRN between treatments for all depths or evaluation dates in this study.

TRL varied significantly between treatments on the evaluation dates, but mostly only towards the end of the 2017 season, particularly for the topsoil (level 4) (Table 3). In agreement with trends in TRN, Mycorrhizae generally had the lowest TRL, although not always significantly so, for all dates and soil depths. Again, seasonal trends for TRL indicated a clustering of treatments into groups with similar mode-of-action, as was identified for TRN i.e., i) the untreated control, ii) Mycorrhiza and iii), *Trichoderma* formulated treatments. In addition, previous studies also mentioned the contribution of biostimulants in increasing nutrient efficiency, promoting growth and increasing yield (Arthur *et al.*, 2016; Bulgari *et al.*, 2019; Calvo *et al.*, 2014; Van Schoor *et al.*, 2008; Van Oosten *et al.*, 2017).

In this study, the effect of biostimulants on root dynamics was studied as an indicator of plant growth. Yet, seasonal increases in TRL followed a more prominent linear pattern, except for the Trich 1 and Trich 2 treatments, that display more distinct high and low values between dates, at all soil depths. Increasing root length and number from this study confirmed typical root biostimulant responses reported by Verbon and Liberman (2016), wherein they reviewed that plant growth promoting rhizobacteria (PGPR) along with ectomycorrhizal fungi, specifically referring to *Trichoderma virens* and *Trichoderma atroviride* markedly affects root growth by stimulating lateral root growth as well as increasing root hair length and density. This could be a possible explanaition for the more prominent results in TRN and TRL as observed using the Trich 1 and Trich 2 treatments.

Calculations based on root numbers, length and diameter measurements provided more information with respect to TRA and TRV. TRA only showed significant differences in the topsoil level, soon after establishment. Mycorrhizae had a significantly higher TRA compared to any of the other treatments, contrary to trends displayed by single indicators. Similar results were reported for TRV values, highlighting the impact of the Mycorrhizae treatment on root development, with significant differences between this treatment and the remainder of the treatments, for several evaluation dates and soil depths. These results are concurrent with the findings of Huang *et al*. (2020), where arbuscular mycorrhizal fungi increased the root surface area and volume in walnut seedlings during a pot trial. Similar results for roots in potted trials were reported when trifoliate oranges under drought stress (Liu *et al.,* 2016) and tea plants in acidic soil (Shao *et al.,* 2018) were inoculated with mycorrhizae.

The correct timing of biostimulant application with refernece to plant stress can influence the effectiveness of biostimulant (Flemming *et al.,* 2019). Applying the optimum dosage is critical, as the effect of the biostimulants could be preventative when applied before the onset of the stress or may operate as remedial when applied concurrent with the stress condition or may act as being curative when applied after stress induction (Andreotti, 2020). The efficacy of a particular biostimulant may also be site or crop specific (Ricci *et al*. 2019). During our study, the treatments applied were once off, at planting, except for Plant Extract that was applied monthly thereafter for six months. Although the correct dosage was applied at establishment according to each protocol, some of the products may have been compromised as it required more frequent applications under standard conditions for tree growth reactions..

As expected, the destructive root study confirmed that thicker roots occur at deeper soil depths. Most of the roots were found at 20 – 40 cm in the soil profile. This analysis also confirmed that root development for young ‘Granny Smith’ apple trees reached 80–100 cm depths, after two seasons, similar to findings by Van Zyl (2016) on ‘Royal Beaut’ apple trees. Despite significant differences between treatments, at specific depths in the various root diameter classes, no clear trends

were observed. This may indicate that the preplant fumigation treatment with 1,3-D and chloropicrin (CP) did not immediately impact root growth to the detrimental effects reported before with MB.

2017/18 season

For the 2017/18 season, only three treatments were selected to continue root studies. Significant differences for TRL were only observed in the topsoil layer at level 3 and 4. The Control consistently showed higher TRN than the Compost and Plant Extract treatments. This trend confirmed observations of the 2016/17 season, but that was only evident for the very topsoil layer (level 4). Tthe Control and Plant Extract treatment showed a consistently higher TRN than was reported for the Compost treatment. Yet, the Control did not always outperform the Plant Extract treatment. TRL followed the same trends as TRN for the 2017/18 season, confirming findings for these treatments from the 2016/17 season.

Significantly higher TRA and TRV values were only noted for the Control compared to the Compost and Plant Extract treatments, for specific dates, and predominantly in the topsoil layer. This finding was also recorded during the 2016/17 season, with respect to level 4, whilst the Plant Extract treatment was consistently higher than the other treatments for the remaining soil depths, although not significantly so in all cases.

Destructive root data

No significant differences regarding averaged total root number occurred between the treatments, but significant differences between treatments occurred with regard to soil depth and root diameter. Most of the fine roots occurred in the top 30 cm, which is concurrent with observations in roots of young apple trees (Van Zyl, 2016). Significant differences between treatments in fine roots followed no clear trend. Control had the highest number of roots with diameters > 2 mm, and at deeper soil levels and differed significantly from the other treatments in this regard. This indicated a different response to treatments, although current information is insufficient to draw final conclusions on this aspect.

Soil probe data

The soil probe data only provided an indication of general conditions that prevailed over the season for a single point in the orchard. Nevertheless, our data indicate the possibility that trees received insufficient irrigation during the first two months after establishment of the orchard, when water was supplied by hand. Thereafter soil moisture throughout the profile increased to such an extent that it allowed root development to proceed to at least 60 cm the first season of growth (root images), and then continue to a depth of 80 cm, by the end of the second season following establishment (destructive root study).

5. CONCLUSION

This study set out to evaluate the effect of a range of biostimulants on the root growth dynamics of young apple trees planted in fumigated soil. Various studies claimed a major benefit of biostimulants to be the alleviation of abiotic stress (Brown and Saa, 2015; Calvo *et al.*, 2014; Du Jardin, 2015; Van Oosten *et al.*, 2017). As fumigation also represents abiotic stress, commercial biostimulant products, from different biostimulant categories, were selected for application on 'Granny Smith' trees at establishment.

Seasonal trends clearly indicated that treatments could be separated onto three distinct groups regarding root dynamics: Mycorrhizae; Trich 1 and Trich 2 treatments; and all other biostimulant treatments evaluated in the 2016/17 season, including the Control. Seasonal TRN and TRL trends for 2016/17 showed low values for the Mycorrhizae treatment in most evaluation dates, which was followed by the Control, Compost, Plant Extract and LAA with increasing order, whilst the highest values were recorded for the Trich 1 and 2 treatments. In contrast, seasonal trends in TRA and TRV illustrated a dominant role of the Mycorrhizae treatment with regard to the extension of root volume and area when compared to the other treatments. Initial TRA and TRV values for Trich 1 and 2 treatments were higher than reported for the Control, Compost, Plant Extract and LAA treatments, however often the final values, after two seasons following establishment, were equal or lower than that of the other treatments, depending on soil depth.

During the 2017/18, significant results in various root dynamics were only obtained for the topsoil layers of levels 3 and 4 for minirhizotron data – indicating the status of the young trees. The root characteristics of the Control consistently differed significantly from those of the Compost and Plant Extract treatments, confirming an extended biostimulant impact on root dynamics in levels 3 or 4, after two seasons. This was supported by results from the destructive study, indicating differential special development of roots and a higher incidence of roots with diameter > 2 mm in the Control treatment – often significant. The effectiveness of biostimulants cannot be guaranteed under all conditions (Ricci *et al.*, 2019) and the difficulty to obtain consistent results on field trials over periods shorter than two seasons have previously been reported (Rouphael *et al.*, 2020). Nevertheless, in our study, there were strong indications that treatments resulted in different reactions with regard to root growth dynamics and supported previous findings, although it was not possible to quantify specific modes of action in the scope of this study.

Based on results from this study, it is not possible yet to recommend a specific biostimulant treatment that can be recommended for a single application at establishment, for a particular root reaction to reduce transplant shock after fumigation. Yet, there is firm evidence that root dynamics were influenced by the biostimulant treatments compared to the Control, even at this limited

application frequency. A better understanding of how the composition of the various treatments relate to the observed root dynamics is required to provide greater insights into the possible mode-of-action that is likely to be the drivers of either a root response, or lack thereof, as was observed over the seasons and with various soil depths. Initial reactions of the roots on the limited application of treatments are encouraging and a change in protocol to allow for additional applications as is commonly practiced, may further enhance the trends. This study contributes to a better understanding of the complexity of soil-plant-microbe interactions during field trials. It provided a strong basis on which further studies with regard to understanding biostimulant effects on and application of possible alternative treatments to address appple replant disease, especially under South-African conditions, can be conducted.

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7. TABLES AND FIGURES

Table 1: Descriptive information on six biostimulant treatments as well as an untreated control as relating to their composition, characteristics, claimed mode-of-action and dosage and method of application as used in an amelioration trial conducted on a newly planted 'Granny Smith'/MM109 apple orchard at Lovenstein farm, Vyeboom during 2016–2018.

Treatment	Product/ Supplier	Composition	Effect / Response	Mode of Action	Application
Control (Fumigation only)	Control	Water	None		Normal Irrigation
Compost (Plant and animal based substrates)	Stellenbosch University	Microbes added to substrates for stimulation of microbial growth	~~-Plant & animal based substrates~~	-Introduction of micro-organisms, nutrients & -Humic-acid substances	-Apply compost at planting
Mycorrhiza	Mycogel from Kimitec/ Wenkem SA (www.wenkem.co.za)	Mycorrhizae	-Increase of root surface -Increases root volume -Increase survival under stress -Provide resistance to pathogens	- Complexes the soil particles -Form soil aggregates which improve nutrient absorption & availability -Promote the absorption of less available nutrients such as P -Create balance with absorption of elements such as Cu & Cl	-0.5ml of product per tree, applied at planting or within 3-4 days of planting -No phosphates or pesticides should be applied within 14 days after application -No mineral fertigation is allowed, only the use of organic fertilizer -Use in transplantation of new crops, before sprouting, but after harvesting
Plant Extract	Aqua Clean SA Sludge Abate Blue Planet SA (www.blueplanet-sa.com)	Plant extracts, PGPB - Bacillus Bacteria (95%), microbes & naturally occurring organic-soluble humates	-Enhance plant growth -Rehabilitate soil -Enhance drought tolerance & nutrient availability to the plant	-Directly as bio-fertilizers or indirectly as bio-pesticides	-150ml of well-mixed product should be added near tree roots -Applied with enough water to spread the product

Table 1 continued: Descriptive information on six biostimulant treatments as well as an untreated control as relating to their composition, characteristics, claimed mode-of-action and dosage and method of application as used in an amelioration trial conducted on a newly planted 'Granny Smith'/MM109 apple orchard at Lovenstein farm, Vyeboom during 2016–2018.

Treatment	Product/ Supplier	Composition	Effect / Response	Mode of Action	Application
L-Amino Acids (LAA)	Aminostim.Xtra® Bioscience Research (www.bsrsa.com)	19 Essential L-Amino acids and oligopeptides	-Supports plant production	-Stimulates the plant metabolism & -providing enhanced resistance against a wide range of stresses	-A liquid formulation mixed with water & applied by means of foliar spray at 0.5L–1L. ha-1), -Drip irrigation at 1.5L to 3L. ha-1 or -Apply in hydroponics at lower rates to young plants and during flowering stages -Applied commercially as a liquid through irrigation every 4 weeks at 25ml per tree or 15L.ha-1
Trichoderma 1 (Trich 1)	Bio-Tricho Liquid Agro-Organics (www.agro-organics.co.za)	Contain 8 different isolates of *Trichoderma*: -*Trichoderma asperellum* (3) -*Trichoderma hamatum* (1) -*Trichoderma atroviride* (2) -*Trichoderma harzianum* (2)	-Stimulates plant and root growth, -Defends against pathogen attacks	-As biocontrol it functions by direct parasitism -or indirectly by activates resistant plant mechanisms, competition, antibiotic production, absorption of nutrients. -Metabolites produced: plant growth hormones (PGH), hydrolytic enzymes, siderophores, and antibiotics	-Tree roots should be submerged in a solution of spore suspension of 400ml.100L-1 product in water -Soak for 10 minutes or longer before transplanting -Commercial soil application at 1L. ha-1
Trichoderma 2 (Trich 2)	Excalibur Gold ABM Africa (www.abm1st.com)	Contains 4 selected species of *Trichoderma* endophytes	-Lives in symbioses with the roots of the plant & -Enhances root growth -Promote nutrient and water uptake by the plant (usually used on vegetable crops) -Increase plant stress tolerance	-As biocontrol by direct parasitism or indirectly by activating plant resistance mechanisms such as antibiotic production, -Absorption of nutrients - Metabolites produced include plant growth hormones & hydrolytic enzymes	-Submerged trees into a solution of the product or as a drench (500 g./ha-1) -No waiting period required; trees can be planted directly after treatment -Recommended as a seed coating (compatible with seed coating fungicides)

Table 2: The total root number count (TRN) for 'Granny Smith'/MM109 apple trees at Lovenstein, Vyeboom, was quantified at 4 different soil depths with a minirhizotron (CID-600) following the application of a range of biostimulant treatments in October of the 2016/17 season. * Indicate missing values due to tube damage.

		Total Root Number: 2016/17 Season								
Soil Level and Depth (cm)	**Treatment**	**15 Dec 2016**	**17 Jan 2017**	**15 Feb 2017**	**14 Mar 2017**	**19 Apr 2017**	**18 May 2017**	**12 Jul 2017**	**30 Aug 2017**	**02 Oct 2017**
1 (45-60 cm)	Control	40.3 ns	32.3 ns	35.3 ns	58.0 ns	60.3 ns	55.7 ns	48.7 ns	63.3 ns	70.0 ab
	Compost	21.7	35.0	35.3	55.7	59.7	60.7	34.7	53.3	55.3 b
	Mycorrhizae	13.0	11.0	9.7	10.7	14.3	14.3	18.7	17.3	12.7 c
	Plant extract	20.3	53.0	62.3	83.7	90.0	91.3	86.3	94.7	96.7 a
	L- Amino Acids (LAA)	10.7	28.5	26.7	56.0	59.0	61.0	47.0	60.7	60.7 ab
	Trichoderma 1 (Trich 1)	42.0	68.7	50.7	82.7	85.0	87.0	47.0	74.7	*
	Trichoderma 2 (Trich 2)	46.3	78.0	82.7	114.0	107.0	109.0	58.0	65.5	66.5 ab
	P-Value	**0.5114**	**0.2031**	**0.1301**	**0.0563**	**0.1202**	**0.1217**	**0.1390**	**0.1161**	**0.0154**
2 (30-45 cm)	Control	33.0 ns	49.3 ab	46.3 ab	70.0 a	70.0 ns	68.0 ns	78.3 ab	94.3 ns	100.3 ab
	Compost	25.0	41.7 bc	44.3 ab	63.0 a	64.3	65.7	31.0 bc	45.3	51.0 bc
	Mycorrhiza	17.7	15.3 c	13.0 c	15.3 b	15.7	14.3	14.7 c	26.3	16.3 c
	Plant extract	30.0	43.0 abc	49.3 ab	80.7 a	86.7	94.3	105.3 a	115.0	120.0 a
	L- Amino Acids (LAA)	14.7	31.7 bc	39.3 bc	54.0 ab	58.3	60.7	28.7 bc	42.3	43.3 bc
	Trichoderma 1 (Trich 1)	68.0	75.7 a	59.7 ab	80.0 a	82.0	69.0	59.5 abc	42.3	*
			ab	ab	a			abc		ab
	Trichoderma 2 (Trich 2)	34.7	59.3	66.7	79.7	87.0	88.0	68.5	58.7	61.0 c
	P-Value	**0.1719**	**0.0419**	**0.0188**	**0.0399**	**0.0529**	**0.1530**	**0.0243**	**0.0777**	**0.0286**
3 (15-30 cm)	Control	34.7 b	59.3 ab	67.3 ab	74.7 abc	77.3 ab	81.7 ab	84.3 ab	98.7 a	96.0 a
	Compost	21.3 b	29.0 bc	34.3 bc	39.7 bcd	41.3 bc	46.0 bc	29.0 bcd	39.7 bc	41.7 b
	Mycorrhiza	9.0 b	12.0 c	9.3 c	10.0 d	8.3 c	7.0 c	15.7 d	16.3 c	13.3 b
	Plant extract	29.3 b	49.3 abc	57.3 ab	69.0 abc	75.0 ab	84.0 ab	90.3 a	98.7 a	102.3 a
	L- Amino Acids (LAA)	6.3 b	20.3 bc	24.7 bc	30.7 cd	26.5 c	23.3 c	17.3 cd	24.7 c	26.0 b
	Trichoderma 1 (Trich 1)	86.3 a	89.0 a	85.7 ab	106.3 a	104.0 a	106.7 a	74.0 abc	85.7 ab	*
	Trichoderma 2 (Trich 2)	40.7 b	58.3 ab	64.3 ab	79.3 ab	104.5 a	106.5 a	49.7 bcd	64.7 abc	66.3 ab
	P-Value	**0.0068**	**0.0301**	**0.0335**	**0.0067**	**0.0029**	**0.0019**	**0.0470**	**0.0131**	**0.017**
4 (0-15 cm)	Control	35.3 a	44.3 a	32.7 ns	48.0 ns	44.5 ab	55.7 a	57.3 a	66.3 a	66.3 a
	Compost	6.3 c	13.7 cd	16.0	21.3	22.0 b	22.3 b	9.3 c	14.3 b	14.7 bc
	Mycorrhiza	6.0 c	8.3 d	9.3	9.3	13.3 b	7.7 b	10.7 bc	15.7 b	10.3 c
	Plant extract	9.0 bc	21.3 bcd	9.0	14.3	19.7 b	21.0 b	12.7 bc	31.0 ab	47.0 ab
	L- Amino Acids (LAA)	2.3 c	8.7 d	10.0	14.0	15.5 b	13.3 b	9.0 c	13.0 b	13.3 bc
	Trichoderma 1 (Trich 1)	27.7 ab	42.7 ab	45.7	54.7	59.7 a	60.7 a	46.0 ab	60.7 a	*
		abc	abc			a	a	abc	b	ab
	Trichoderma 2 (Trich 2)	21.0	31.7	30.7	50.7	75.0	74.5	27.0	42.0	43.3 c
	P-Value	**0.0198**	**0.0101**	**0.0836**	**0.0663**	**0.0124**	**0.0038**	**0.0388**	**0.0262**	**0.0187**

ns Different letters used to show significant values when differences occurred at a 5% confidence level (P<0.005)

Table 3: The total root length for 'Granny Smith'/MM109 trees at Lovenstein, Vyeboom, as quantified with a minirhizotron (CID-600) and Rootsnap software following the application of a range of biostimulant treatments, at four soil depths (levels), during the 2016/17 season. * Indicate missing values due to tube damage.

		Total Root Length (mm): 2016/17 season								
Soil Level & Depth (cm)	**Treatment**	**15 Dec 2016**	**01 Jan 2017**	**15 Feb 2017**	**14 Mar 2017**	**19 Apr 2017**	**18 May 2017**	**12 Jul 2017**	**30 Aug 2017**	**02 Oct 2017**
1 (45-60 cm)	Control	897.6 ns	869.0 ns	994.0 ns	1988.0 ns	2081.0 ns	1938.0 ns	1863.0 ns	2426.0 ns	2619.0 ab
	Compost	949.5	1628.0	1640.0	2804.0	2970.0	3028.0	1738.0	2651.0	2752.3 ab
	Mycorrhiza	551.7	608.0	671.0	696.0	873.0	773.0	1117.0	1083.0	876.7 b
	Plant Extract	891.0	2483.0	2959.0	3717.0	4014.0	4061.0	3873.0	4313.0	4398.6 a
	L- Amino Acids (LAA)	391.9	1017.0	1022.0	2448.0	2588.0	2696.0	2038.0	2949.0	2965.1 ab
	Trichoderma 1 (Trich 1)	1755.5	2919.0	2290.0	3723.0	3839.0	3908.0	2054.0	3147.0	*
	Trichoderma 2 (Trich 2)	1756.1	3354.0	3618.0	5119.0	4843.0	4927.0	2242.0	2650.0	2710.1 ab
	P-Value	**0.3607**	**0.1872**	**0.1119**	**0.0811**	**0.1768**	**0.1544**	**0.2788**	**0.2208**	**0.0369**
2 (30-45 cm)	Control	1053.3 ns	1607.9 ns	1542.6 cd	2485.1 a	2532.4 ab	2483.0 ns	2551.4 ab	3103.0 ns	3323.0 ab
	Compost	1116.1	1981.9	2084.5 abc	3082.1 a	3172.1 a	3228.0	1519.0 b	2250.0	2569.0 b
	Mycorrhiza	795.5	973.5	799.9 d	813.3 b	876.5 b	856.0	959.2 b	1553.0	1064.0 b
	Plant Extract	1298.3	1899.0	2313.3 abc	3391.2 a	3635.6 a	3879.0	4484.6 a	5006.0	5239.0 a
	L- Amino Acids (LAA)	735.8	1516.6	1856.0 bc	2617.4 a	2828.5 a	2959.0	1539.2 b	2195.0	2253.0 b
	Trichoderma 1 (Trich 1)	2840.3	3336.4	2571.7 ab	3542.6 a	3663.4 a	3281.0	2888.0 ab	2081.0	*
	Trichoderma 2 (Trich 2)	1450.9	2615.2	2956.5 a	3728.8 a	3723.0 a	3816.0	2833.4 ab	2473.0	2569.0 b
	P-Value	**0.2023**	**0.1058**	**0.0108**	**0.0218**	**0.0317**	**0.1484**	**0.0333**	**0.2177**	**0.0474**
3 (15-30 cm)	Control	1369.1 bc	2302.1 abc	2605.0 ab	3101.7 ab	3260.9 ab	3222.3 bc	3472.0 ab	4120.6 ab	4016.7 ab
	Compost	995.6 bc	1490.2 bc	1713.0 abc	2059.8 bc	2164.3 bc	2355.9 cd	1201.0 c	1850.6 cd	2033.7 bcd
	Mycorrhiza	502.0 bc	756.7 c	558.8 c	738.1 c	617.8 c	525.8 e	1001.0 c	1097.3 c	769.4 d
	Plant Extract	1420.8 bc	2552.8 ab	2992.7 ab	3651.3 a	3905.0 a	4205.2 ab	4518.0 a	4990.6 a	5180.2 a
	L- Amino Acids (LAA)	376.1 c	1153.7 bc	1433.2 bc	1687.9 bc	1367.4 c	1261.5 de	936.0 c	1394.5 cd	1523.9 cd
	Trichoderma 1 (Trich 1)	3087.6 a	3351.0 a	3235.1 a	4080.7 a	4090.5 a	4126.6 ab	2752.0 abc	3293.5 abc	*
	Trichoderma 2 (Trich 2)	1665.6 b	2763.0 ab	3091.2 a	3886.8 a	4861.6 a	4972.0 a	2040.0 bc	2840.6 bcd	2974.9 bc
	P-Value	**0.0077**	**0.048**	**0.0239**	**0.0026**	**0.001**	**0.0003**	**0.0220**	**0.0094**	**0.0068**
4 (0-15 cm)	Control	926.3 ns	1325.0 ab	1089.7 ns	1650.2 abc	1675.7 ab	1963.3 ab	2051.2 a	2435.6 a	2444.3 a
	Compost	222.3	517.5 bc	614.4	895.1 bc	923.1 b	935.6 bc	405.7 c	672.5 c	683.1 bc
	Mycorrhiza	339.3	537.2 bc	381.7	489.3 c	563.3 b	335.3 cd	632.4 bc	952.6 bc	551.5 c
	Plant Extract	391.7	1028.2 abc	237.7	538.7 c	824.2 b	855.7 bc	447.5 c	1188.9 abc	1536.8 abc
	L- Amino Acids (LAA)	67.4	308.2 c	380.0	557.7 c	539.4 b	496.7 cd	360.3 c	543.0 c	561.1 c
	Trichoderma 1 (Trich 1)	1116.1	1731.6 a	1906.2	2302.9 a	2510.6 a	2516.7 a	1545.6 ab	2096.3 ab	*
	Trichoderma 2 (Trich 2)	857.5	1374.0 ab	1364.4	2110.9 ab	3086.7 a	3031.1 a	880.5 bc	1682.7 abc	1763.7 ab
	P-Value	**0.0710**	**0.0448**	**0.0507**	**0.0488**	**0.0150**	**0.0023**	**0.0177**	**0.0410**	**0.0156**

ns Different letters used to show significant values when differences occurred at a 5% confidence level (P<0.005)

Table 4: The total root area for 'Granny Smith'/MM109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap software following the application of biostimulant treatments during the 2016/17 season. * Indicate missing values due to tube damage. [ns] Different letters used to show significant values a P<0.005

Total Root Area (mm^2): 2016/17 season																					
Soil Level & Depth (cm)	**Treatment**	**15 Dec 2016**		**01 Jan 2017**		**15 Feb 2017**		**14 Mar 2017**		**19 Apr 2017**		**18 May 2017**		**12 Jul 2017**		**30 Aug 2017**		**02 Oct 2017**	
			n		n		n		n		n		n		n		n		n
1 (45-60 cm)	Control	1369.0	ns	1478.0	ns	1668.0	ns	3796.0	ns	3897.0	ns	3658.0	ns	3108.0	ns	4315.0	ns	4677.0	ns
	Compost	1883.0		3143.0		3157.0		5476.0		5805.0		5950.0		3074.0		4967.0		5126.0	
	Mycorrhiza	4241.0		9789.0		5478.0		8324.0		9678.0		8909.0		15757.0		17241.0		23729.0	
	Plant Extract	1503.0		4346.0		5152.0		6459.0		6889.0		6946.0		6350.0		7078.0		7215.0	
	L- Amino Acids (LAA)	773.0		1764.0		1799.0		4131.0		4320.0		4508.0		3480.0		5070.0		5103.0	
	Trichoderma 1	3393.0		5578.0		4192.0		7019.0		7264.0		7381.0		3448.0		5417.0		*	
	Trichoderma 2	2935.0		6011.0		6576.0		9274.0		8754.0		8973.0		3636.0		4401.0		4509.0	
	P-Value	**0.7617**		**0.2129**		**0.3305**		**0.8342**		**0.7527**		**0.9092**		**0.1064**		**0.1401**		**0.1299**	
			n		n		n		n		n		n		n		n		n
2 (30-45 cm)	Control	1912.0	ns	2904.0	ns	2703.0	ns	4122.0	ns	4148.0	ns	4181.0	ns	3514.0	ns	4544.0	ns	5028.0	ns
	Compost	2128.0		3968.0		4148.0		5848.0		6040.0		6136.0		3068.0		4700.0		5383.0	
	Mycorrhiza	13171.0		14839.0		10198.0		11121.0		14267.0		13517.0		15830.0		13688.0		20287.0	
	Plant Extract	2952.0		3284.0		4112.0		5776.0		6152.0		6451.0		7784.0		8572.0		9024.0	
	L- Amino Acids (LAA)	1379.0		2611.0		3131.0		4578.0		4887.0		5067.0		2786.0		3992.0		4086.0	
	Trichoderma 1	4771.0		5724.0		5082.0		7059.0		7241.0		6298.0		5417.0		3974.0		*	
	Trichoderma 2	2708.0		4919.0		5417.0		7050.0		7652.0		7873.0		5750.0		5052.0		5270.0	
	P-Value	**0.1545**		**0.1492**		**0.1558**		**0.3474**		**0.2806**		**0.3800**		**0.3390**		**0.6480**		**0.2912**	
			n		n		n		n		n		n		n		n		n
3 (15-30 cm)	Control	2503.0	ns	4786.0	b	5404.0	ns	6078.0	ns	6342.0	ns	6299.0	ns	5630.0	ns	6846.0	ns	6711.0	ns
	Compost	1798.0		3145.0	b	3599.0		4369.0		4526.0		5062.0		2639.0		4056.0		4451.0	
	Mycorrhiza	5805.0		13956.0	a	11295.0		12201.0		5347.0		9394.0		15490.0		22014.0		13972.0	
	Plant Extract	2298.0		4468.0	b	5190.0		6507.0		6931.0		7551.0		8149.0		8911.0		9301.0	
	L- Amino Acids (LAA)	636.0		1862.0	b	2267.0		2783.0		2511.0		2208.0		1575.0		2405.0		2670.0	
	Trichoderma 1	5691.0		6424.0	b	6412.0		7919.0		7937.0		7972.0		4980.0		6162.0		*	
	Trichoderma 2	3088.0		5097.0	b	5783.0		7407.0		9221.0		9448.0		3898.0		5474.0		5809.0	
	P-Value	**0.6528**		**0.0472**		**0.0673**		**0.3957**		**0.2721**		**0.2737**		**0.0856**		**0.0714**		**0.1660**	
							n		n		n		n		n		n		n
4 (0-15 cm)	Control	1368.0	b	2046.0	b	1838.0	ns	2745.0	ns	3392.0	ns	3328.0	ns	3868.0	ns	4632.0	ns	4628.0	ns
	Compost	404.0	b	1060.0	b	1404.0		1950.0		1992.0		2146.0		1126.0		1754.0		1777.0	
	Mycorrhiza	5236.0	a	12685.0	a	*		9224.0		9924.0		5841.0		6170.0		18034.0		11282.0	
	Plant Extract	572.0	b	1503.0	b	387.0		943.0		1360.0		1413.0		657.0		1959.0		2530.0	
	Trichoderma & Amino Acids (LAA)	121.0	b a	610.0	b	719.0		1013.0		1067.0		1067.0		767.0		1215.0		1264.0	
	Trichoderma 1	2476.0	b	3620.0	b	3947.0		4753.0		5128.0		5136.0		2651.0		4204.0		*	
	Trichoderma 2	1362.0	b	2496.0	b	2566.0		4390.0		6603.0		6299.0		1645.0		3298.0		3435.0	
	P-Value	**0.0276**		**0.0413**		**0.1420**		**0.1001**		**0.1295**		**0.3129**		**0.1073**		**0.3254**		**0.2636**	

Table 5: The total root volume for 'Granny Smith'/MM109 at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap software following the application of biostimulant treatments during 2016/17. * Indicate missing values due to tube damage. [ns] Different letters used to show significant values a P<0.005

		Total Root Volume (mm^3): 2016/17 season								
Soil Level & Depth	**Treatment**	**15 Dec 2016**	**1 Jan 2017**	**15 Feb 2017**	**14 Mar 2017**	**19 Apr 2017**	**18 May 2017**	**12 Jul 2017**	**30 Aug 2017**	**2 Oct 2017**
1 (45-60 cm)	Control	226.0[ns]	261.0 [b]	282.0 [b]	782.0[ns]	769.0[ns]	722.0[ns]	506.0[ns]	744.0 [b]	806.0 [b]
	Compost	333.0	535.0 [b]	537.0 [b]	946.0	1004.0	1035.0	513.0	857.0 [b]	877.0 [b]
			17845.		10541.	11825.	10941.	25341.	27623.	59733.
	Mycorrhiza	3345.0	0 [a]	6038.0 [a]	0	0	0	0	0 [a]	0 [a]
	Plant Extract	240.0	667.0 [b]	777.0 [b]	966.0	1016.0	1022.0	884.0	982.0 [b]	1000.0 [b]
	L- Amino Acids (LAA)	134.0	284.0 [b]	287.0 [b]	615.0	635.0	663.0	513.0	744.0 [b]	749.0 [b]
	Trichoderma 1	618.0	1010.0 [b]	692.0 [b]	1188.0	1232.0	1244.0	545.0	868.0 [b]	*
	Trichoderma 2	450.0	971.0 [b]	1080.0 [b]	1528.0	1422.0	1478.0	542.0	689.0 [b]	705.0 [b]
	P-Value	**0.5336**	**0.0003**	**0.0062**	**0.3721**	**0.1088**	**0.4154**	**0.1168**	**0.0179**	**0.0456**
2 (30-45 cm)	Control	332.0[ns]	506.0 [b]	438.0[ns]	619.0 [b]	618.0 [b]	645.0 [b]	458.0[ns]	633.0[ns]	729.0 [b]
	Compost	377.0	720.0 [b]	744.0	995.0 [b]	1034.0 [b]	1050.0 [b]	570.0	883.0	1009.0 [b]
	Mycorrhiza	23709	22087 [a]	15557	18407 [a]	23717 [a]	20612 [a]	29520	23885	35083 [a]
	Plant Extract	631.0	521.0 [b]	737.0	966.0 [b]	1014.0 [b]	1042.0 [b]	1304.0	1401.0	1481.0 [b]
	L- Amino Acids (LAA)	229.0	395.0 [b]	460.0	698.0 [b]	735.0 [b]	752.0 [b]	451.0	640.0	653.0 [b]
	Trichoderma 1	788.0	946.0 [b]	1007.0	1334.0 [b]	1356.0 [b]	1097.0 [b]	978.0	726.0	*
	Trichoderma 2	480.0	874.0 [b]	937.0	1243.0 [b]	1460.0 [b]	1509.0 [b]	1119.0	980.0	1026.0 [b]
	P-Value	**0.2179**	**0.0186**	**0.0573**	**0.0432**	**0.0280**	**0.0343**	**0.3144**	**0.4323**	**0.0395**
3 (15-30 cm)					1178.0[n]	1220.0[n]				
	Control	454.0[ns]	1015.0 [b]	1153.0 [b]	[s]	[s]	1264.0 [b]	870.0[ns]	1066.0 [b]	1053b [b]
	Compost	287.0	697.0 [b]	783.0 [b]	936.0	953.0	1100.0 [b]	548.0	812.0 [b]	894.0 [b]
	Mycorrhiza	9129	25218 [a]	20139 [a]	19029	5886.	16179 [a]	25076	38974 [a]	24707 [a]
	Plant Extract	343.0	902.0 [b]	1003.0 [b]	1238.0	1293.0	1420.0 [b]	1511.0	1604.0 [b]	1680.0 [b]
	L- Amino Acids (LAA)	88.0	255.0 [b]	302.0 [b]	395.0	400.0	333.0 [b]	217.0	351.0 [b]	397.0 [b]
	Trichoderma 1	1096.0	1257.0 [b]	1298.0 [b]	1547.0	1543.0	1531.0 [b]	856.0	1140.0 [b]	*
	Trichoderma 2	571.0	915.0 [b]	1053.0 [b]	1408.0	1678.0	1717.0 [b]	708.0	972.0 [b]	1043.0 [b]
	P-Value	**0.5110**	**0.0044**	**0.0029**	**0.0751**	**0.1360**	**0.0242**	**0.0586**	**0.0128**	**0.0240**
4 (0-15 cm)	Control	194.0 [b]	311.0[ns]	310.0[ns]	439.0 [b]	636.0[ns]	552.0[ns]	771.0[ns]	906.0[ns]	895.0 [ns]
	Compost	64.0 [b]	188.0	371.0	462.0 [b]	467.0	619.0	524.0	648.0	652.0
	Mycorrhiza	7665 [a]	27276	13490	17809 [a]	17732	11482	9177	29616	20613
	Plant Extract	67.0 [b]	180.0	52.0	165.0 [b]	214.0	223.0	78.0	295.0	362.0
	L- Amino Acids (LAA)	18.0 [b]	118.0	132.0	172.0 [b]	203.0	238.0	151.0	246.0	257.0
	Trichoderma 1	543.0 [b]	803.0	855.0	1000.0 [b]	1059.0	1060.0	416.0	873.0	*
	Trichoderma 2	183.0 [b]	535.0	566.0	979.0 [b]	1505.0	1397.0	284.0	578.0	596.0
	P-Value	**0.0374**	**0.0807**	**0.1559**	**0.0312**	**0.0649**	**0.3714**	**0.4082**	**0.2850**	**0.1306**

Table 6: The total root number (TRN) for 'Granny Smith'/MM109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap software following the application of three different biostimulant treatments at four soil depths (levels) during the 2017/18 season. * Indicate missing values due to tube damage.

Total Root Number: 2017/18 season											
Soil Level & Depth	**Treatment**	**28 Nov 2017**	**05 Jan 2018**	**31 Jan 2018**	**27 Feb 2018**	**11 Mar 2018**	**11 Apr 2018**	**23 May 2018**	**20 Jun 2018**	**02 Aug 2018**	**31 Aug 2018**
1 (45-60cm)	Control	33.3 ns	37.0 ns	45.3 ns	47.0 ns	25.0 ns	64.0 ns	53.0 ns	53.0 ns	65.5 ns	66.0 ns
	Compost	42.0	43.0	31.7	35.0	36.5	63.0	45.3	45.7	46.0	38.5
	Plant Extract	15.0	26.0	19.3	21.0	*	33.0	35.0	35.3	35.3	35.3
	P-Value	**0.3708**	**0.5545**	**0.3943**	**0.3617**	**0.8315**	**0.0578**	**0.6173**	**0.6299**	**0.3363**	**0.3270**
2 (30-45 cm)	Control	27.0 ns	36.7 ns	42.7 ns	44.3 ns	15.0 ns	64.0 ns	49.0 ns	50.7 ns	66.5 ns	67.5 ns
	Compost	38.0	44.0	32.3	34.0	50.5	17.0	43.0	44.0	44.3	44.3
	Plant Extract	18.0	32.0	27.3	27.3	*	39.0	40.3	41.0	41.0	41.0
	P-Value	**0.6033**	**0.8356**	**0.7669**	**0.7279**	**0.0773**	**0.2376**	**0.9062**	**0.8847**	**0.4130**	**0.3900**
3 (15-30 cm)	Control	50.7 ns	60.3 ns	66.0 ns	69.0 ns	39.0 ns	92.0 ns	74.7 ns	74.7 ns	94.0 ns	94.0 ns
	Compost	18.5	22.0	16.0	19.3	21.7	*	23.0	23.7	23.7	23.7
	Plant Extract	16.0	31.0	23.7	24.0	*	36.0	37.3	37.3	37.3	37.3
	P-Value	**0.1452**	**0.1496**	**0.0533**	**0.0573**	*	**0.0330**	**0.0647**	**0.0676**	**0.0075**	**0.0075**
4 (0-15 cm)	Control	20.3 ns	31.7 a	35.0 a	38.3 a	* ns	43.3 ns	20.3 a	21.7 a	25.0 a	27.5 a
	Compost	3.5	8.0 b	6.7 b	8.3 b	9.3	*	6.0 b	6.3 b	6.7 b	6.7 b
	Plant Extract	7.0	17.5 b	15.0 b	16.0 b	*	18.7	4.0 b	4.0 b	4.0 b	1.3 b
	P-Value	**0.0833**	**0.0031**	**0.0007**	**0.0006**	*	**MAR***	**0.0449**	**0.0326**	**0.0294**	**0.0220**

ns Different letters used to show significant values when differences occurred at a 5% confidence level (P<0.005)

Table 7: The total root length (TRL; mm^2) for 'Granny Smith'/MM109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap software following the application of three biostimulant treatments at four soil depths (levels) during the 2017/18 season. * Indicate missing values due to tube damage.

		Total Root Length (mm): 2017/18 season									
Soil Level & Depth	**Treatment**	**28 Nov 2017**	**05 Jan 2018**	**31 Jan 2018**	**27 Feb 2018**	**11 Mar 2018**	**11 Apr 2018**	**23 May 2018**	**20 Jun 2018**	**02 Aug 2018**	**31 Aug 2018**
1 (45-60 cm)	Control	1336.6 ns	1554.8 ns	2011.6 ns	2096.1 ns	862.0 ns	3027.1 ns	2377.0 ns	2383.0 ns	3107.0 ns	3134.0 ns
	Compost	2247.1	2325.9	1698.1	1876.3	1815.0	3799.9	2490.0	2534.0	2571.0	2318.0
	Plant Extract	470.1	1002.8	753.3	781.9	*	1383.0	1545.0	1564.0	1564.0	1567.0
	P-Value	**0.3554**	**0.4146**	**0.4669**	**0.3964**	**0.7226**	**0.0670**	**0.6342**	**0.6358**	**0.4112**	**0.4951**
2 (30-45 cm)	Control	1438.2 ns	1903.0 ns	2204.3 ns	2289.2 ns	780.9 ns	3395.6 ns	2571.1 ns	2627.0 ns	3507.2 ns	3527.3 ns
	Compost	1866.0	2227.8	1738.4	1977.7	2772.3	1509.9	2581.8	2623.9	2636.9	2636.9
	Plant Extract	932.3	1287.7	1059.5	1098.7	*	1689.8	1760.1	1796.6	1809.1	1809.1
	P-Value	**0.6943**	**0.6338**	**0.5152**	**0.4508**	**0.1039**	**0.0969**	**0.6102**	**0.6021**	**0.1355**	**0.1318**
3 (15-30 cm)	Control	2293.7 ns	2830.0 a	3084.2 ns	3218.0 ns	1604.7 ns	4476.1 a	3529.4 ns	3553.9 ns	4593.1 a	4593.1 a
	Compost	838.6	1041.9 b	805.2	1087.2	1244.9	*	1327.3	1344.7	1344.7 b	1350.2 b
	Plant Extract	657.3	1259.9 b	974.8	998.2	*	1757.7 b	1859.5	1859.5	1859.5 b	1859.5 b
	P-Value	**0.1635**	**0.0182**	**0.0558**	**0.0627**	**0.2595**	**0.0057**	**0.0825**	**0.0847**	**0.0006**	**0.0006**
4 (0-15 cm)	Control	973.6 ns	1634.4 a	1791.0 a	1981.1 a	*	2287.2 a	767.1 ns	827.2 a	1016.5 a	1124.9 a
	Compost	197.1	381.0 b	308.0 b	354.5 b	*	* b	251.6	278.0 b	287.3 b	293.7 b
	Plant Extract	363.1	786.1 c	694.7 b	772.5 b	*	907.3 b	186.1	186.1 b	186.1 b	92.7 b
	P-Value	**0.0887**	**0.0012**	**0.0004**	**0.0005**		**0.0136**	**0.0632**	**0.0434**	**0.0100**	**0.0082**

ns Different letters used to show significant values when differences occurred at a 5% confidence level (P<0.005)

Table 8: The total root area (TRA; mm^2) for 'Granny Smith'/M109 trees at Loevenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap software following the application of three biostimulant treatments at four soil depths (levels) during the 2017/18 season. *Indicate missing values due to tube damage.

		Total Root Area (mm^2): 2017/18 season									
Soil Level & Depth	**Treatment**	**28 Nov 2017**	**5 Jan 2018**	**31 Jan 2018**	**27 Feb 2018**	**11 Mar 2018**	**11 Apr 2018**	**23 May 2018**	**20 Jun 2018**	**02 Aug 2018**	**31 Aug 2018**
1 (45-60 cm)	Control	2044.0 ns	2520.0 ns	3252.0 ns	3388.0 ns	1292.0 ns	4906.0 ns	3808.0 ns	3823.0 ns	5011.0 ns	5052.0 ns
	Compost	4408.0	4540.0	3462.0	4159.0	3873.0	7770.0	5172.0	5223.0	5370.0	5350.0
	Plant Extract	834.0	1535.0	1148.0	1183.0	*	2104.0	2389.0	2413.0	2413.0	2417.0
	P-Value	**0.2635**	**0.2883**	**0.4073**	**0.2316**	**0.4340**	**0.0724**	**0.3512**	**0.3518**	**0.2504**	**0.3667**
2 (30-45 cm)	Control	1887.0 ns	2596.0 ns	3138.0 ns	3264.0 ns	1091.4 ns	4888.0 ns	3686.0 ns	3804.0 ns	5100.0 ns	5164.0 ns
	Compost	3590.0	4437.0	3544.0	4098.0	5411.7	3833.0	5273.0	5436.0	5491.0	5491.0
	Plant Extract	1501.0	1923.0	1675.0	1744.0	*	2557.0	2660.0	2710.0	2728.0	2728.0
	P-Value	**0.2277**	**0.2569**	**0.4822**	**0.3061**	**0.1179**	**0.2069**	**0.2354**	**0.2241**	**0.0880**	**0.0837**
3 (15-30 cm)	Control	3506.9 ns	4778.0 ns	5278.0 ns	5522.0 ns	3237.9 ns	7387.0 ns	6022.0 ns	6056.0 ns	7571.0 a	7571.0 a
	Compost	1643.1	2257.0	1999.0	2825.0	3233.0	*	3454.0	3556.0	3556.0 b	3624.0 b
	Plant Extract	869.8	2695.0	2105.0	2183.0	*	3430.0	3578.0	3578.0	3578.0 b	3578.0 b
	P-Value	**0.0918**	**0.3661**	**0.1573**	**0.1805**	**0.9965**	**0.0536**	**0.2075**	**0.2153**	**0.0254**	**0.0292**
4 (0-15 cm)	Control	1744.6 ns	3479.4 a	3792.9 a	4232.2 a	*	4848.6 a	1274.0 ns	1393.5 ns	1651.8 ns	1855.5 ns
	Compost	707.0	1114.4 b	908.8 b	1014.2 b	*	* b	876.4	951.0	964.1	1029.1
	Plant Extract	701.0	1563.5 b	1370.5 b	1555.9 b	*	1843.2 b	380.6	380.6	380.6	220.2
	P-Value	**0.2457**	**0.0035**	**0.0021**	**0.0006**		**0.0089**	**0.2073**	**0.1638**	**0.1223**	**0.0850**

ns Different letters used to show significant values when differences occurred at a 5% confidence level (P<0.005)

Table 9: The total root volume (TRV; mm^3) for 'Granny Smith'/MM109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap software following the application of three biostimulant treatments at four soil depths (levels) during the 2017/18 season. * Indicate missing values due to tube damage.

		Total Root Volume (mm^3): 2017/18 season									
Soil Level & Depth	**Treatment**	**28 Nov 2017**	**05 Jan 2018**	**31 Jan 2018**	**27 Feb 2018**	**11 Mar 2018**	**11 Apr 2018**	**23 May 2018**	**20 Jun 2018**	**02 Aug 2018**	**31 Aug 2018**
1 (45-60 cm)	Control	271.6 ^{ns}	387.4 ^{ns}	484.2 ^{ns}	502.4 ^{ns}	165.0 ^{ns}	728.1 ^{ns}	557.6 ^{ns}	560.0 ^{ns}	739.1 ^{b}	744.1 ^{ns}
	Compost	807.5	830.0	685.8	953.6	921.2	1460.9	1095.6	1098.5	1145.6 ^{a}	1271.8
	Plant Extract	137.0	204.1	150.6	154.0	*	274.2	315.3	317.7	317.7 ^{b}	318.3
	P-Value	**0.2197**	**0.2556**	**0.2688**	**0.0637**	**0.1955**	**0.0759**	**0.0767**	**0.0767**	**0.0427**	**0.0505**
2 (30-45 cm)	Control	225.9 ^{b}	323.5 ^{ns}	411.8 ^{ns}	426.4 ^{ns}	130.5 ^{ns}	637.7 ^{ns}	475.5 ^{b}	500.0 ^{b}	678.0 ^{b}	695.7 ^{b}
	Compost	643.0 ^{a}	808.4	655.5	771.3	952.6	851.4	970.9 ^{a}	1029.2 ^{a}	1047.6 ^{a}	1047.6 ^{a}
	Plant Extract	216.7 ^{b}	260.1	250.9	260.5	*	361.2	373.3 ^{b}	378.6 ^{b}	380.7 ^{b}	380.7 ^{b}
	P-Value	**0.0415**	**0.0774**	**0.3098**	**0.1123**	**0.1135**	**0.2077**	**0.0433**	**0.0438**	**0.0332**	**0.0311**
3 (15-30 cm)	Control	459.5 ^{ns}	746.1 ^{ns}	833.3 ^{ns}	871.8 ^{ns}	648.8 ^{ns}	1082.4 ^{ns}	940.3 ^{ns}	971.5 ^{ns}	1105.3 ^{ns}	1105.3 ^{ns}
	Compost	281.4	431.9	536.4	752.6	840.0	*	911.0	985.5	985.5	1052.4
	Plant Extract	99.2	572.7	449.4	470.4	*	647.1	663.9	663.9	663.9	663.9
	P-Value	**0.0628**	**0.7050**	**0.5484**	**0.5149**	**0.2524**	**0.3770**	**0.6175**	**0.5343**	**0.4688**	**0.4806**
4 (0-15 cm)	Control	273.7 ^{ns}	660.4 ^{ns}	712.8 ^{ns}	799.3 ^{a}	*	911.9 ^{a}	177.4 ^{ns}	198.2 ^{ns}	221.4 ^{ns}	253.8 ^{ns}
	Compost	306.2	384.5	298.4	321.7 ^{b}	*	* ^{b}	414.5	433.1	434.6	487.2
	Plant Extract	134.0	291.2	247.4	287.3 ^{b}	*	347.4 ^{b}	82.6	82.6	82.6	49.4
	P-Value	**0.7189**	**0.1668**	**0.0540**	**0.0220**		**0.0230**	**0.2027**	**0.1976**	**0.2625**	**0.2315**

^{ns} Different letters used to show significant values when differences occurred at a 5% confidence level (P<0.005)

Table 10. Total root number of three replicates for 'Granny Smith/MM109' trees per 10cm soil depth according to the methodology of Böhm (1979) and Van Zyl (2016) for the untreated Control, Compost and Plant Extract treatments at Lovenstein, Vyeboom for July 2018.

	Root number per soil depth											
Treatment	10	20	30	40	50	60	70	80	90	100	Tot. Root No.	SE
Control	17	14	31	16	12	9	6	5	0	0	159	13.93
Compost	17	13	34	22	12	1	2	4	3	0	152	16.22
Plant extract	4	23	31	22	15	9	4	3	0	3.3	145	29.61

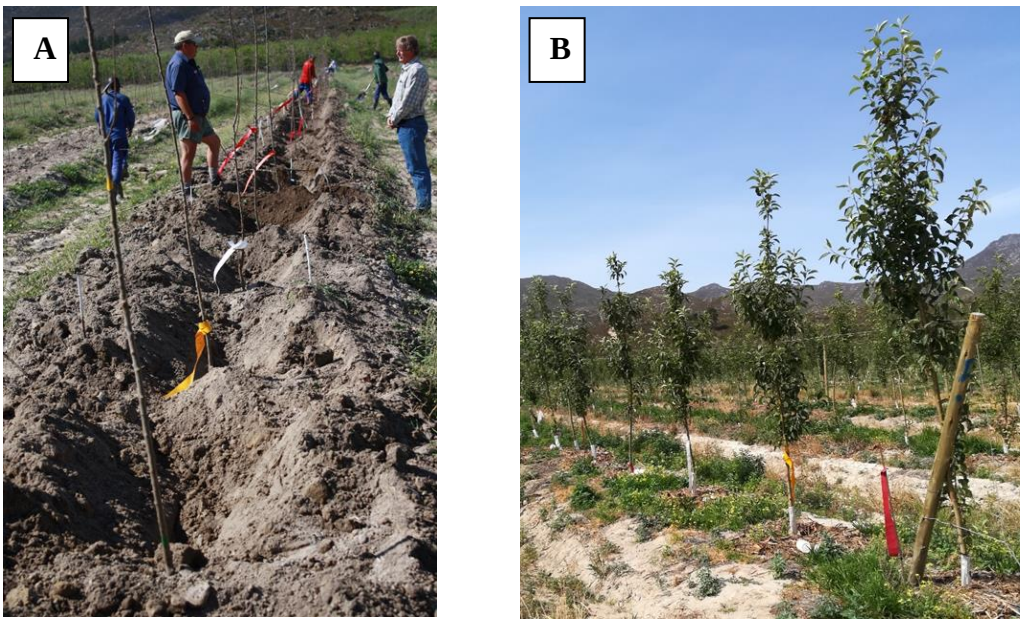


Figure 1: (A) A young 'Granny Smith' /MM 109 apple orchard being prepared for planting in October 2016 on Lovenstein farm, in Vyeboom, Western Cape (South Africa) and (B) established 14-month-old saplings, in January 2018, with a planting distance of 5m x 2m.

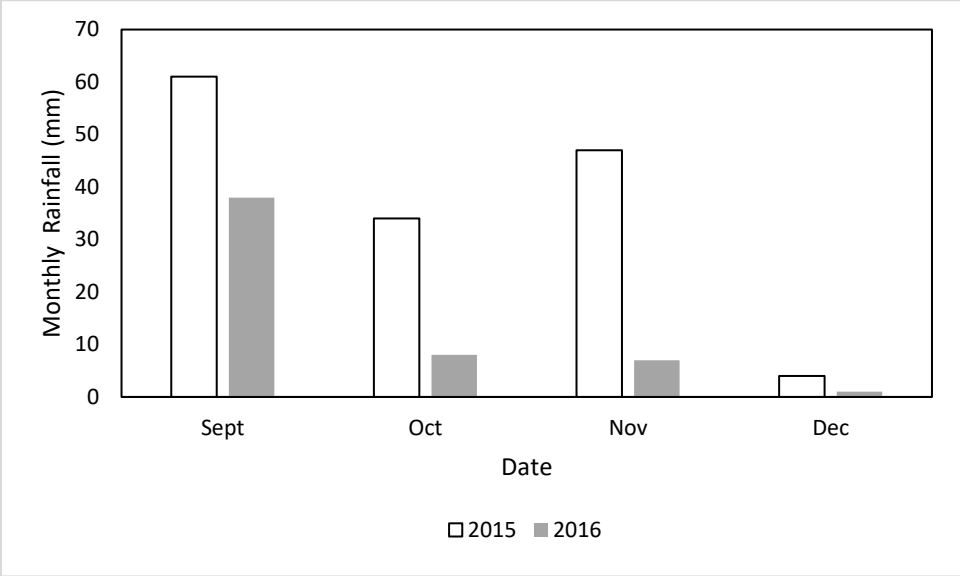


Figure 2: Monthly rainfall (mm) for the months of September to December for 2015 and 2016 at Vyeboom, Western Cape as supplied by Hortec (Unit D45, Olive Grove Industrial Estate, 5 Old Paardevlei Rd, Somerset West, 7130).

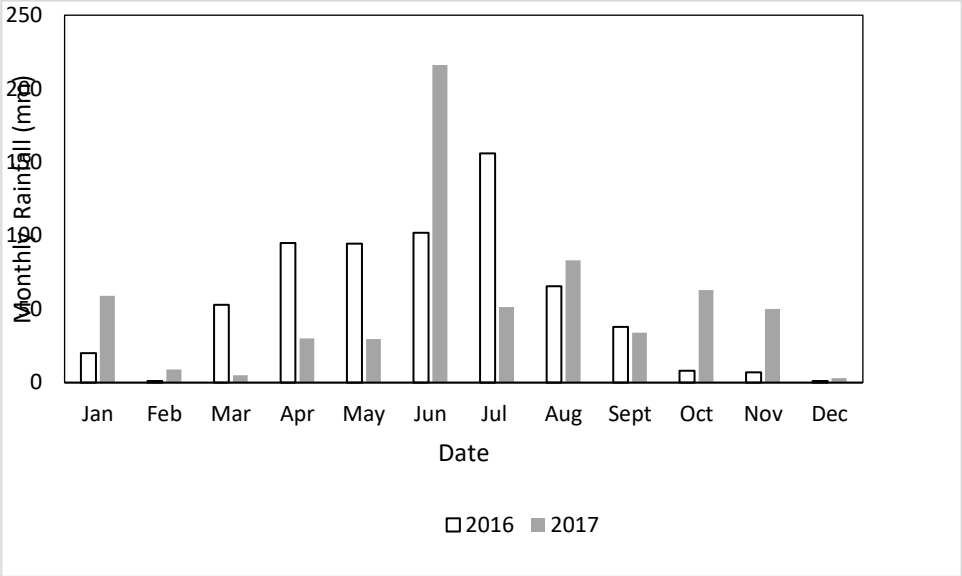


Figure 3: Monthly rainfall (mm) for Jan to Dec (2016 and 2017) in Vyeboom, Western Cape (South Africa) as supplied by Hortec (Unit D45, Olive Grove Industrial Estate, 5 Old Paardevlei Rd, Somerset West, 7130).

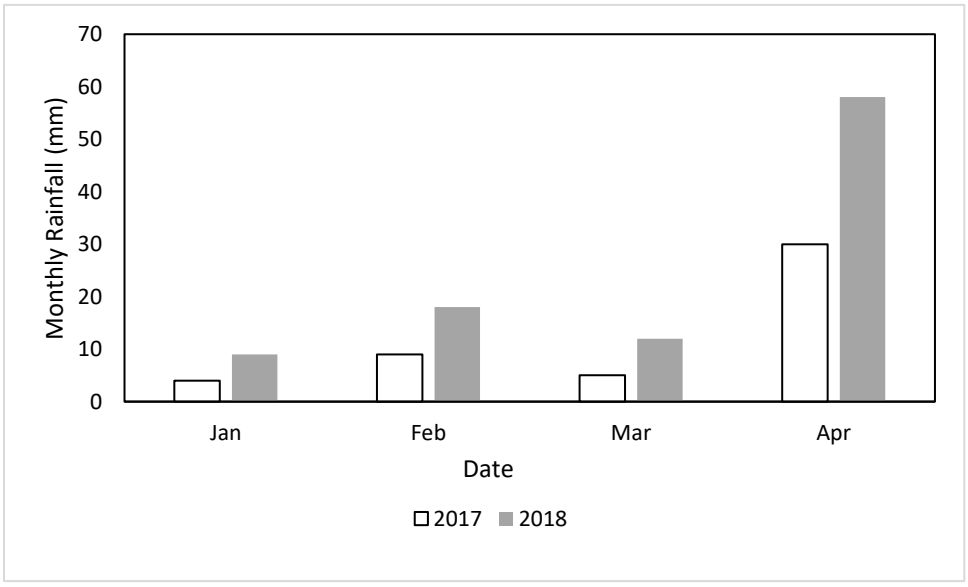


Figure 4: Monthly rainfall (mm) for January to April (2017 and 2018) for Vyeboom, Western Cape (South Africa) as provided by Hortec (Unit D45, Olive Grove Industrial Estate, 5 Old Paardevlei Rd, Somerset West, 7130).

Figure 5: Transparent poly-ethylene tubes of 1m in length was inserted at a 45˚ angle, 10 cm from the 'Granny Smith'/MM109 apple tree trunks for capturing images using a minirhizotron and CID-600 Root scanner. After installation (A), the above ground section of the tube was sealed with a removable lid (B).

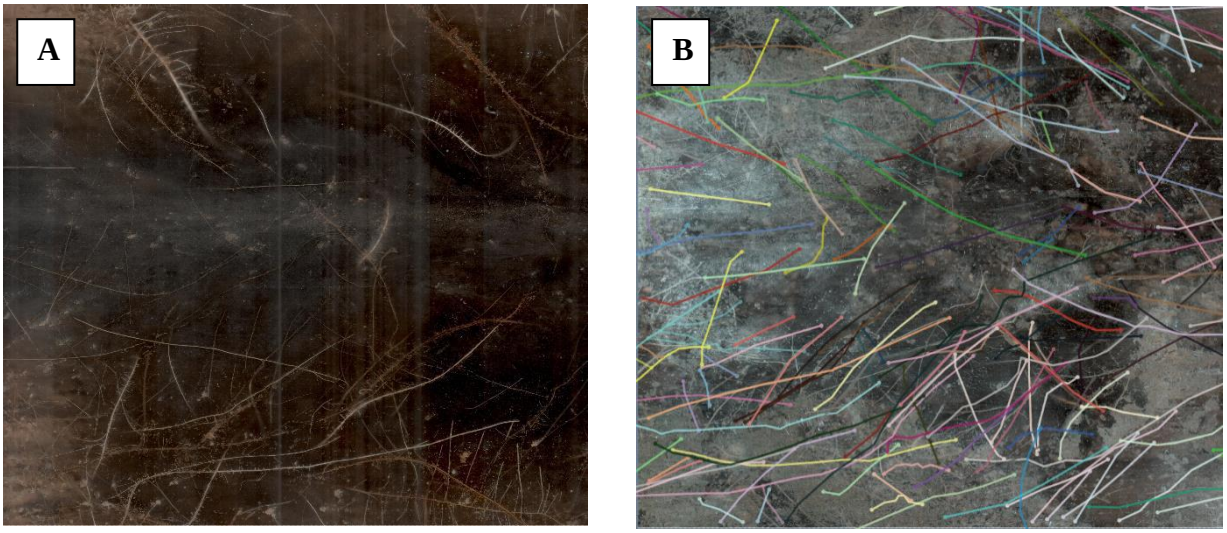


Figure 6: A 360˚ image of young 'Granny Smith'/MM109 apple tree roots that received the Plant Extract treatment. The image was recorded at a depth of 60 cm (A in May 2017 on Lovenstein farm, Vyeboom captured using a minirhizotron and CID-600 Root scanner. (B) A root image created by *RootSnap* software at 40 cm soil depth, following quantification of roots.

Figure 7: A root profile of a young ‘Granny Smith’/MM109 tree on Lovenstein farm, Vyeboom as revealed with destructive root analyses conducted on 17 July 2018, using a 100 cm x 100 cm grid.

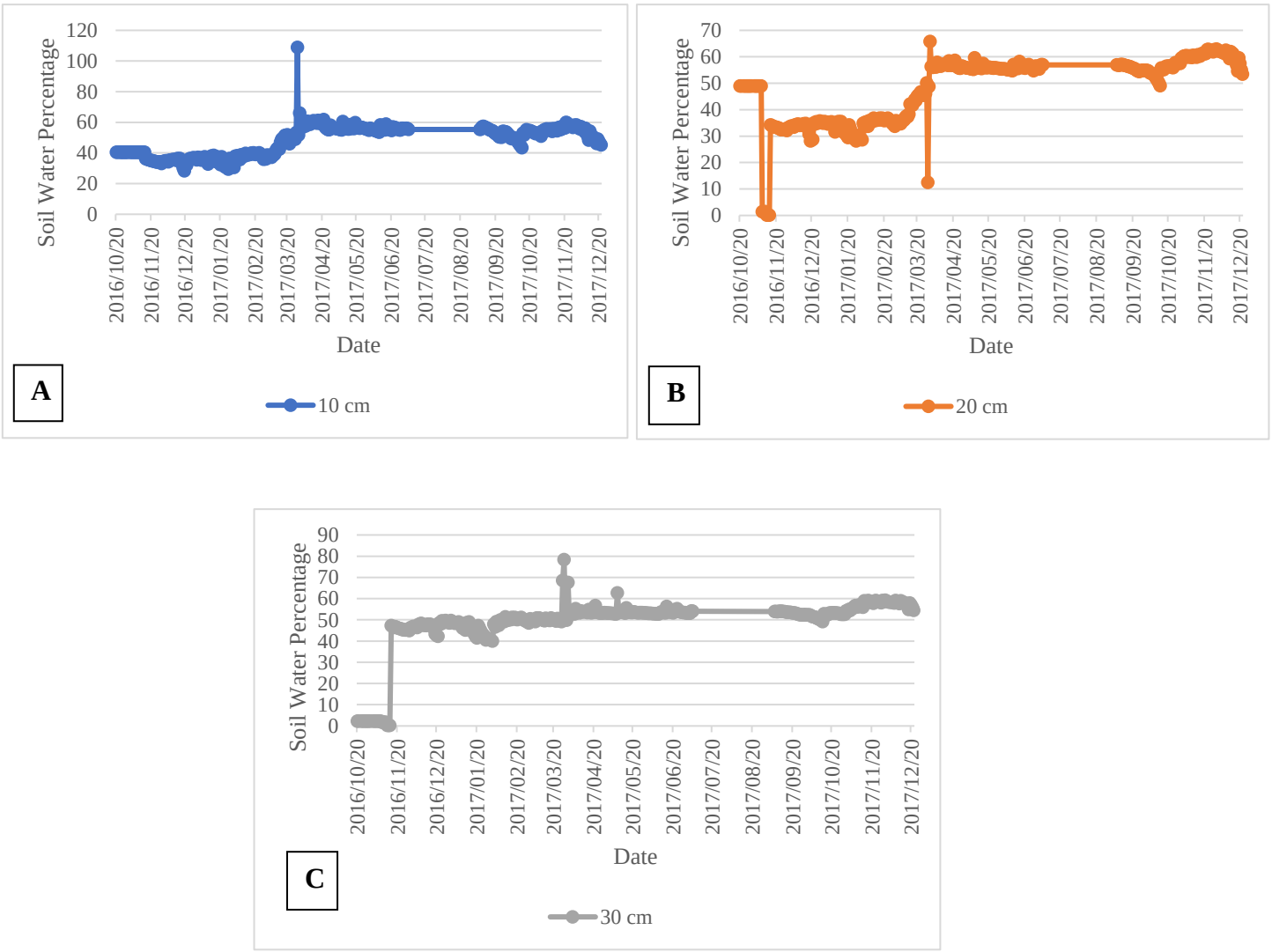


Figure 8: The monthly soil moisture percentage (%) from Oct 2016 to Dec 2017, for 10 cm (A), 20 cm (B) and 30 cm (C) soil depth for a young 'Granny Smith'/MM109 apple orchard on Lovenstein farm, Vyeboom, using a DFM Soil Moisture Logging probe.

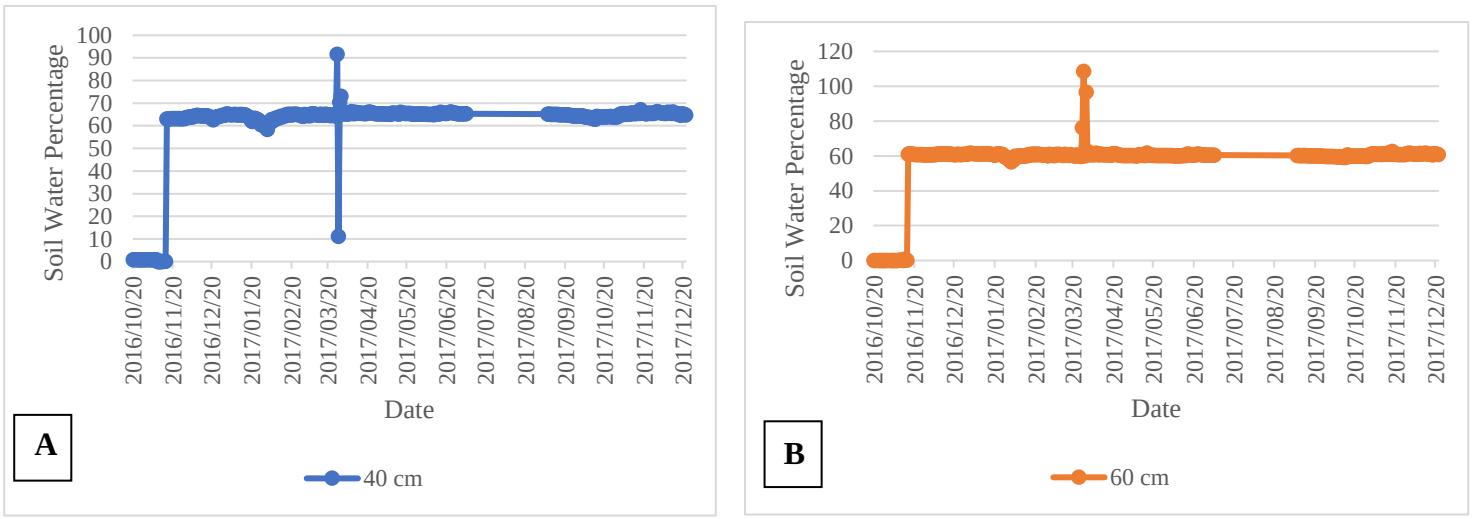


Figure 9: The monthly soil moisture percentage for 40 cm (A) and 60 cm (B) soil depth on Lovenstein farm, Vyeboom, from Oct 2016 to Dec 2017, for a young 'Granny Smith'/MM109 apple orchard, using a DFM Soil Moisture Logging probe.

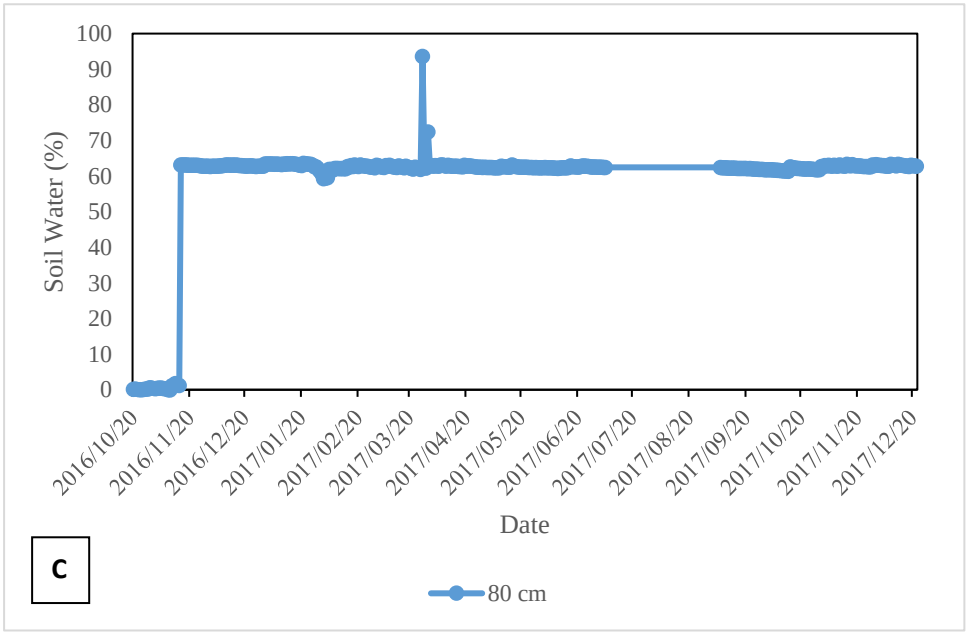


Figure 10: The monthly soil moisture percentage (%) at 80 cm soil depth on Lovenstein farm, Vyeboom, from Oct 2016 to Dec 2017, for a young 'Granny Smith'/MM109 apple orchard, using a DFM Soil Moisture Logging probe.

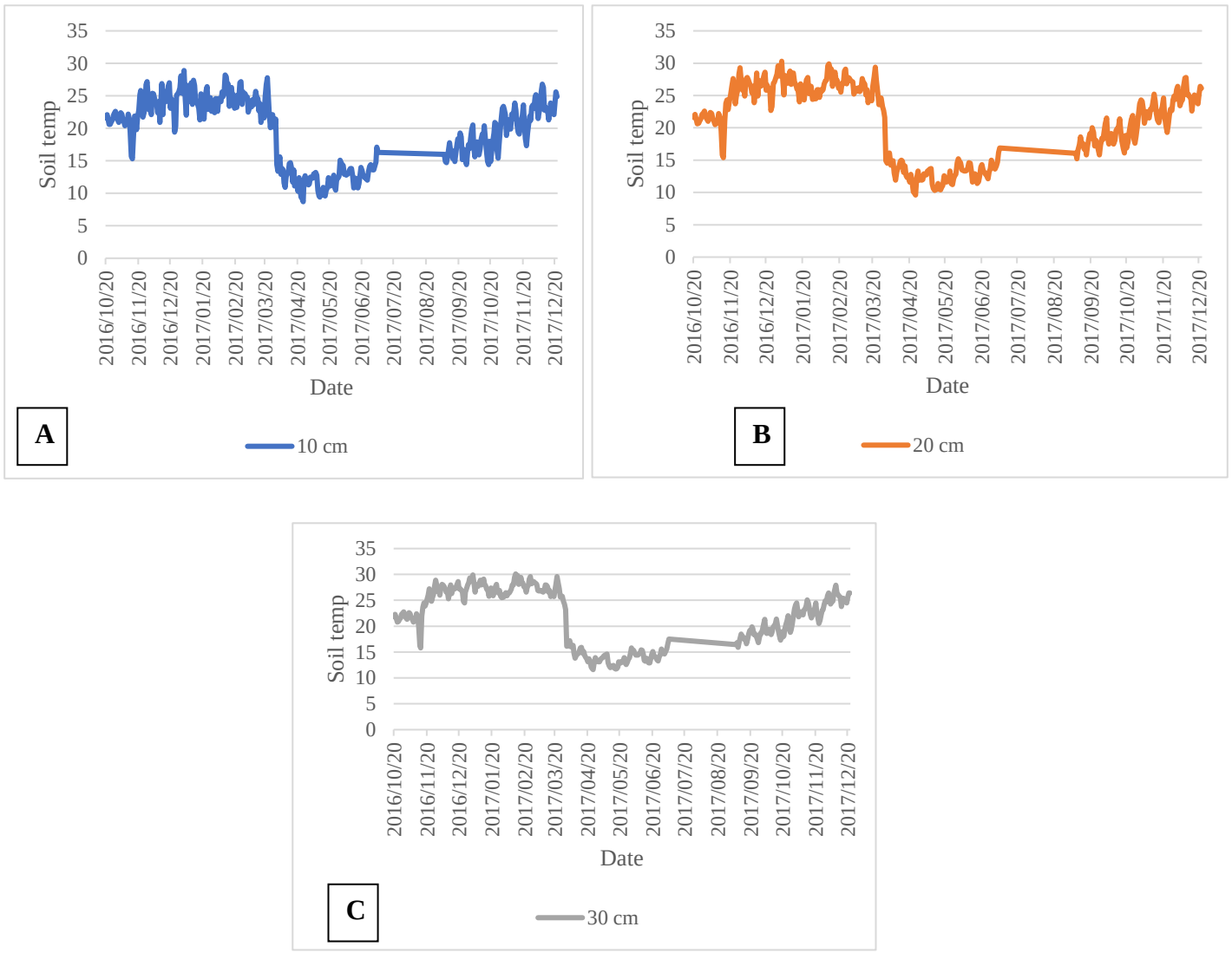


Figure 11: The monthly soil temperatures (°C) for 10 cm (A), 20 cm (B) and 30 cm (C) soil depth on Lovenstein farm, Vyeboom, from Oct 2016 to Dec 2017, for a young 'Granny Smith'/MM109 apple orchard, using a DFM Soil Moisture Logging probe.

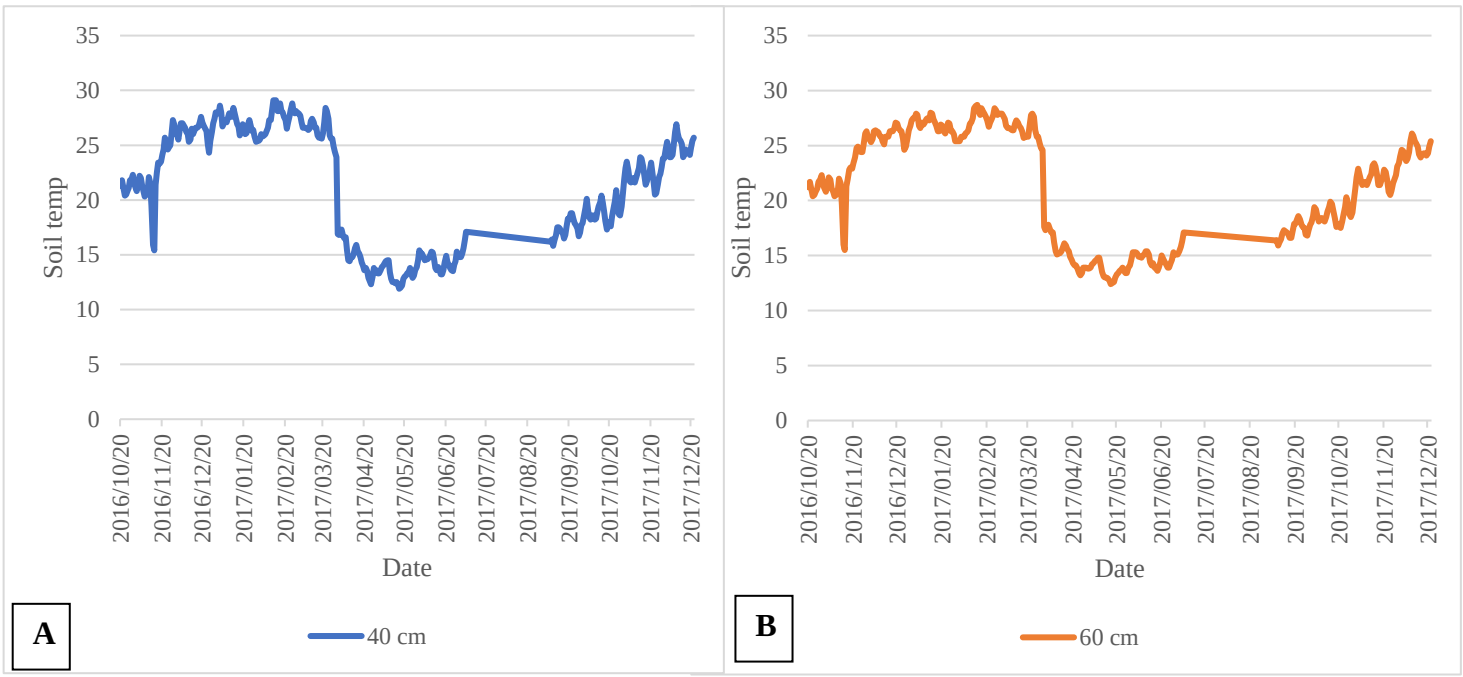


Figure 12: The monthly soil temperatures (°C) for 40 cm (A) and 60 cm (B) soil depth on Lovenstein farm, Vyeboom, from Oct 2016 to Dec 2017, for a young 'Granny Smith'/MM109 apple orchard, using a DFM Soil Moisture Logging probe.

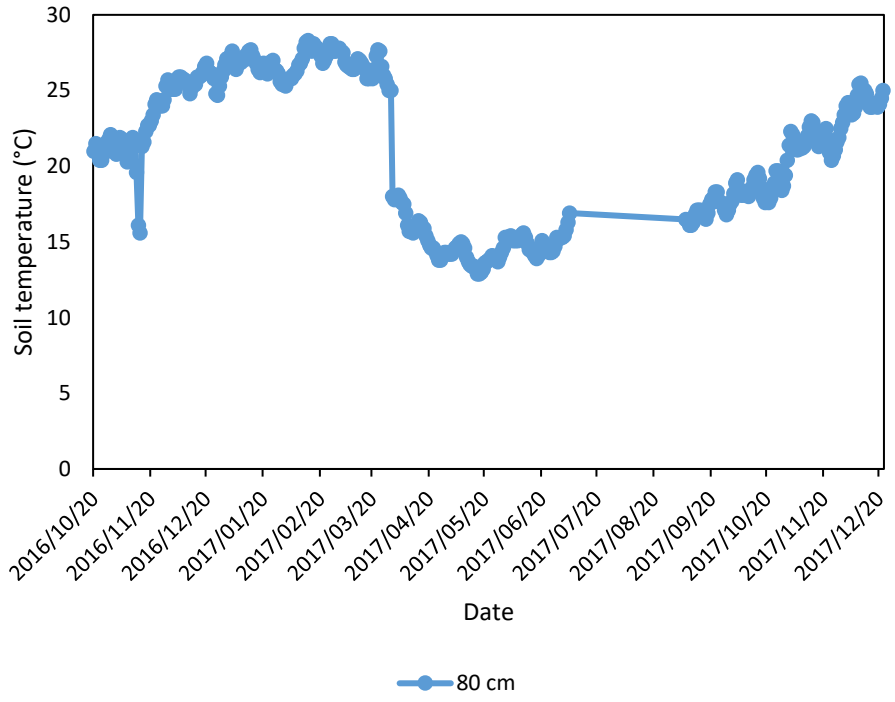


Figure 13: The monthly soil temperatures (°C) for 80 cm soil depth on Lovenstein farm, Vyeboom, from Oct 2016 to Dec 2017, for a young 'Granny Smith'/MM109 apple orchard, using a DFM Soil Moisture Logging probe.

Control											
Tree 1											
Profile width (cm)											
Profile depth (cm)		10	20	30	40	50	60	70	80	90	100
	10			∘∘∘	∘∘∘∘∘∘◯	∘∘∘∘	∘∘∘∘∘	∘∘∘∘∘∘∘	∘∘∘		∘
	20		∘∘	∘∘∘∘	∘∘∘∘∘∘∘	∘∘∘∘∘∘∘∘∘	∘∘∘∘∘∘	∘∘	∘∘	∘∘∘∘	∘∘∘∘∘∘∘
	30		∘∘	∘∘∘	∘∘∘	∘∘∘∘∘◯	∘∘◯		∘	∘∘∘∘	∘∘∘∘∘∘∘∘
	40				∘∘∘◯	∘∘	∘∘●		∘	∘∘	∘∘∘∘∘∘
	50			∘	∘∘	∘		∘∘	∘	∘∘	∘∘
	60	∘		∘	∘∘	∘∘	∘∘∘	∘∘∘	∘∘	∘∘	
	70				∘	∘∘	∘∘				
	80										
	90										
	100										

Tree 2											
Profile depth (cm)		10	20	30	40	50	60	70	80	90	100
	10		∘∘	∘	∘∘						
	20	∘∘∘	∘∘	∘∘∘∘∘∘	∘∘∘∘∘∘∘∘∘∘●	∘∘∘∘∘∘∘∘●	∘∘∘●◯	∘		∘	∘∘●
	30	∘∘	∘	∘	∘∘∘∘∘∘●	∘∘∘∘∘●◯◯	∘∘∘∘∘∘●	∘		∘	∘∘∘
	40	∘	∘∘∘∘	∘∘∘∘	∘∘	∘	∘∘●	∘	∘	∘∘	∘
	50	∘●	∘∘	∘∘	∘∘				∘∘∘	∘∘∘∘∘	∘∘∘
	60	∘∘∘∘∘∘∘	∘	∘∘∘∘∘∘	∘∘∘∘∘∘∘	∘∘∘∘	∘∘∘∘	∘	∘∘∘	∘	∘∘∘∘
	70	∘	∘∘	∘∘∘∘	∘∘∘	∘∘∘∘∘∘	∘∘	∘∘∘	∘	∘	
	80		∘	∘∘		∘	∘∘			∘	∘∘
	90										
	100										

Tree 3											
Profile depth (cm)		10	20	30	40	50	60	70	80	90	100
	10				∘∘∘∘	∘∘∘∘∘∘	∘∘∘∘	∘∘			
	20	∘∘	∘∘∘	∘∘∘∘∘	∘∘	∘∘∘∘∘∘∘	∘∘∘∘∘∘∘∘∘●●●◯	∘∘∘∘∘∘∘◯	∘∘∘∘●●◯	∘	
	30	∘∘	∘∘∘	∘∘∘	∘∘∘	∘∘∘∘∘∘∘	∘∘∘∘●	∘∘∘●●◯	∘∘		
	40			∘∘∘	∘		∘∘∘		∘	∘∘	∘
	50	∘	∘∘	∘						∘	∘
	60						∘		∘		
	70	∘∘∘	∘	∘		∘∘∘	∘∘∘	∘∘	∘	∘∘∘∘	∘
	80		∘						∘∘∘		∘∘
	90										
	100										

Root Size	Symbol
<2mm	∘
2mm-5mm	○
5mm-10mm	◯
1cm-2cm	◯
2cm-5cm	◯

Figure 14: Visual representation of root size and distribution following destructive root analysis of young 'Granny Smith'/MM109 trees for the Control treatment in a 100 cm^2 soil profile on Lovenstein, on 17 July 2018.

Compost

Tree 1

Profile width (cm)

Profile depth (cm)	10	20	30	40	50	60	70	80	90	100
10				∘						
20	∘∘∘	∘	∘∘∘∘∘	∘∘∘∘∘○○◯	∘∘∘∘∘◯	○			∘∘	∘∘∘∘∘
30	∘∘∘∘∘∘∘∘∘	∘∘∘	∘∘◯◯	∘∘∘∘∘∘◯	∘∘∘∘○○○	∘∘∘∘∘∘◯		∘∘∘	∘∘∘∘∘∘∘	∘∘∘
40		∘∘	∘∘	∘∘∘∘	∘∘∘∘∘	∘∘∘∘∘		∘∘∘	∘∘∘	∘∘∘
50			∘		∘∘∘					
60			∘	∘	∘∘○	∘				
70		∘∘	∘∘∘	∘∘∘∘	∘∘∘					
80	○		∘∘∘	∘∘		∘∘∘∘				○○
90	○	∘∘∘∘			○	∘∘∘				∘
100										

Tree 2

Profile depth (cm)	10	20	30	40	50	60	70	80	90	100
10		∘∘	∘∘	∘∘∘∘	∘∘∘∘∘∘∘∘∘	∘∘∘∘∘∘∘	∘	∘∘∘		
20		∘	∘∘∘∘∘∘∘	∘∘∘∘∘∘	∘∘∘∘∘∘∘∘∘◯	∘∘∘∘∘◯	∘∘∘	∘∘∘		∘∘∘∘∘ ∘∘
30	∘∘	∘∘	∘∘	∘∘∘	∘∘∘∘	∘∘∘	∘∘			∘
40	∘∘	∘∘∘	∘∘∘	∘∘	∘∘	∘	∘∘	∘∘ ○	∘∘	∘∘○
50	∘∘	∘∘∘	∘	∘∘∘	∘∘	∘	∘			
60	∘∘		∘∘							
70										
80										
90										
100										

Tree 3

Profile depth (cm)	10	20	30	40	50	60	70	80	90	100
10			∘∘∘	∘∘∘∘∘∘	∘∘∘∘∘∘∘∘◯	∘∘		∘∘		
20	∘∘◯	∘∘∘∘∘	∘∘∘◯	∘∘∘∘	∘∘◯◯◯◯◯	∘∘∘∘◯	∘∘∘∘	∘∘∘∘∘∘∘∘		∘∘
30	∘∘∘∘	∘∘∘∘∘∘∘◯	∘∘∘∘∘∘◯	∘∘∘∘	∘∘∘	∘∘∘∘	∘∘∘			
40	∘∘∘∘		∘∘			∘∘∘	∘∘∘	∘∘	∘	
50	∘∘∘∘	∘∘∘∘∘∘	∘∘			∘∘	∘	∘∘		∘
60	∘	∘		∘∘∘∘∘	∘∘∘∘	∘∘∘∘∘∘	∘∘∘	∘	∘∘	
70	∘∘∘∘	∘∘∘∘∘	∘	∘∘∘∘∘	∘∘∘∘	∘∘∘∘	∘∘			
80										
90										
100										

Figure 15: Visual representation of root size and distribution of young 'Granny Smith'/MM109 trees following destructive root analysis for the Compost treatment in a 100 cm^2 soil profile on Lovenstein, on 17 July 2018.

Plant Extract											
Tree 1											
Profile width (cm)											
Profile depth (cm)		10	20	30	40	50	60	70	80	90	100
	10		○	○○○○○	○○○○○	○○○○	○	○○○	○○○	○○	○○○
	20	○○○○◯	○○○○○	○○○○◯	○○○○○	○○○○○○○○○ ○	○○○○◯	○○○◯	○○○○○◯	○○○○	○○○○○
	30	○○○○○	○○○○○○	○○○○○○○◯	○○○○○○○○	○○○○○○○○○ ○○○	○○○○○○	○○○	○○○○	○	○
	40	○○○○○	○○	○○	○○○○	○○○○	○○○◯	○○○○	○○	○○	
	50		○○		○○○	○○○	○○	○○	○		
	60	○○						○○○○○○	○○		
	70			○○	○		○○○○	○○			
	80		○	○		○○		○○○○		○	
	90										
	100										
Tree 2											
Profile depth (cm)		10	20	30	40	50	60	70	80	90	100
	10			○○○○	○○	○○○○	○	○			
	20	○○		○○○	○○○	○○	○○○		○○		
	30			○	○○	○○○○	○			○○	
	40		○○○○	○	○○○	○○○◯	○○		○○○○	○○○○○○	
	50	○○○○	○○○○○	○○	○○○	○○	○○○○	○○	○○○○○○○	○○○○	
	60			○○	○○○	○○	○	○○	○○○	○○○	
	70								○		
	80										
	90										
	100										

Tree 3											
Profile depth (cm)		10	20	30	40	50	60	70	80	90	100
	10	○	○	○○◯	○○○	○○○○○◯◯◯	○○○○○	○○○	○○	○○○	
	20	○	○○○○	○○○	○○○○○○○	○○○○○○	○○○○○○○	○○○○○○	○○		
	30	○○	○○	○○○○	○○	○○○	○○○	○○○○○	○	○○○○○	○○
	40	○○○	○○○○○○◯	○○					○		
	50										
	60										
	70		○	○							
	80										
	90										
	100	○	○	○○○	○	○○○					○

Root Size	Symbol
<2mm	◦
2mm-5mm	○
5mm-10mm	◯
1cm-2cm	◯
2cm-5cm	◯

Figure 16: Visual representation of root size and distribution of young 'Granny Smith'/MM109 trees, following destructive root analysis for the Plant Extract treatment in a 100 cm^2 soil profile on Lovenstein, on 17 July 2018.

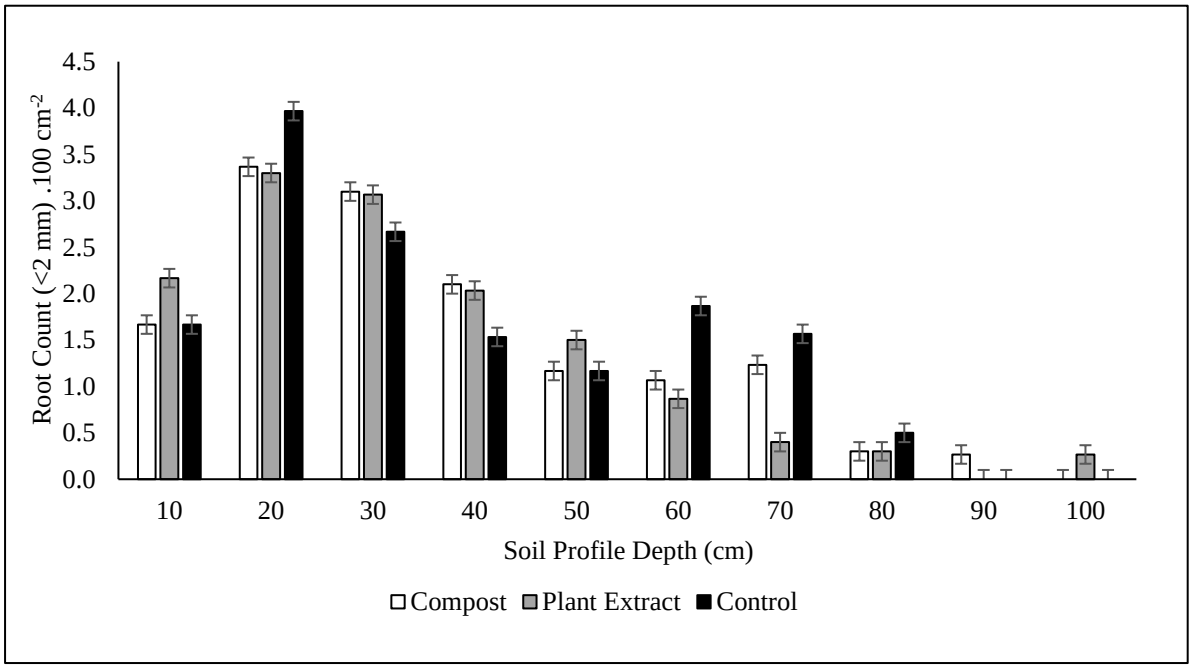


Figure 17: Average root count.100 cm^{-2} ± SE for roots with a diameter <2 mm observed during destructive root analysis of young 'Granny Smith'/MM109 apple trees following the application of three biostimulant treatments (Compost, Plant Extract and Control) with establishment.

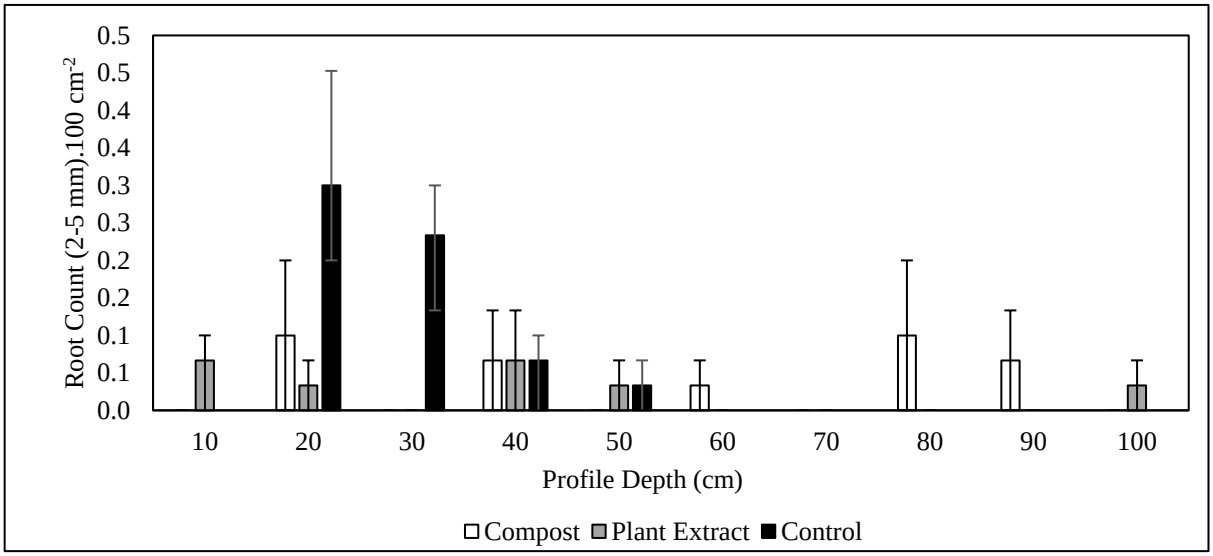


Figure 18: Average root count.100 cm^{-2} ± SE for roots with a diameter of 2-5 mm observed during destructive root analysis of young 'Granny Smith'/MM109 apple trees following the application of three biostimulant treatments (Compost, Plant Extract and Control) with establishment.

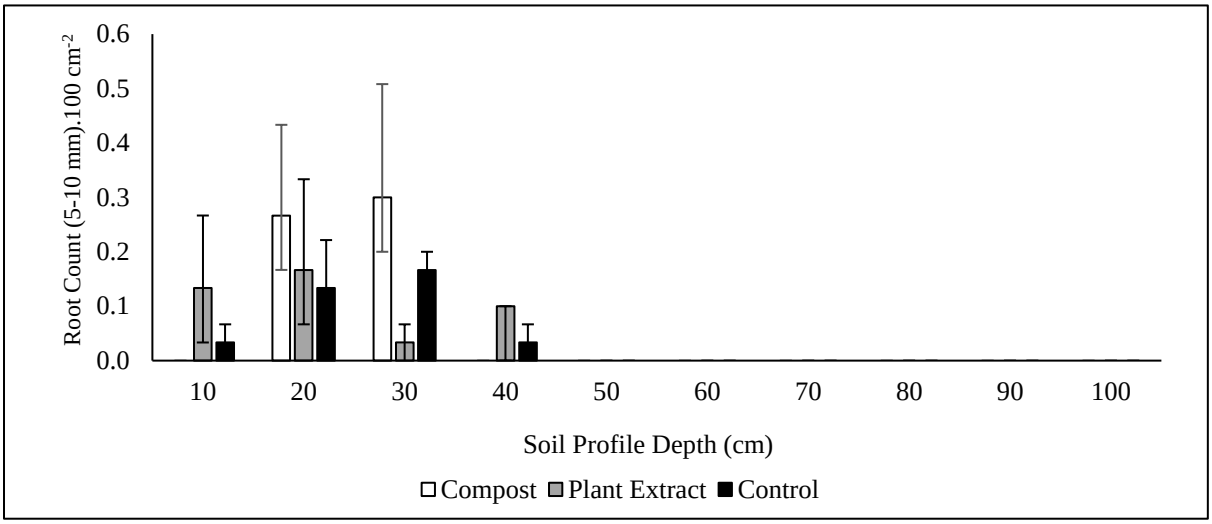


Figure 19: Average root count.100 cm^{-2} ± SE for roots with a diameter of 5-10 mm observed during destructive root analysis of young 'Granny Smith'/MM109 apple trees following the application of three biostimulant treatments (Compost, Plant Extract and Control) with establishment.

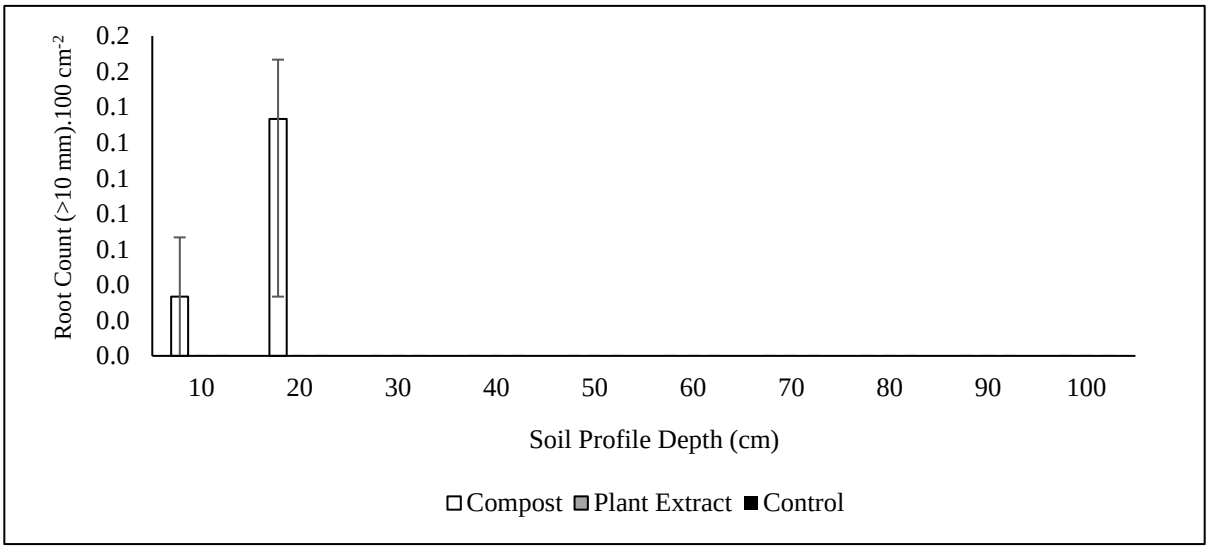
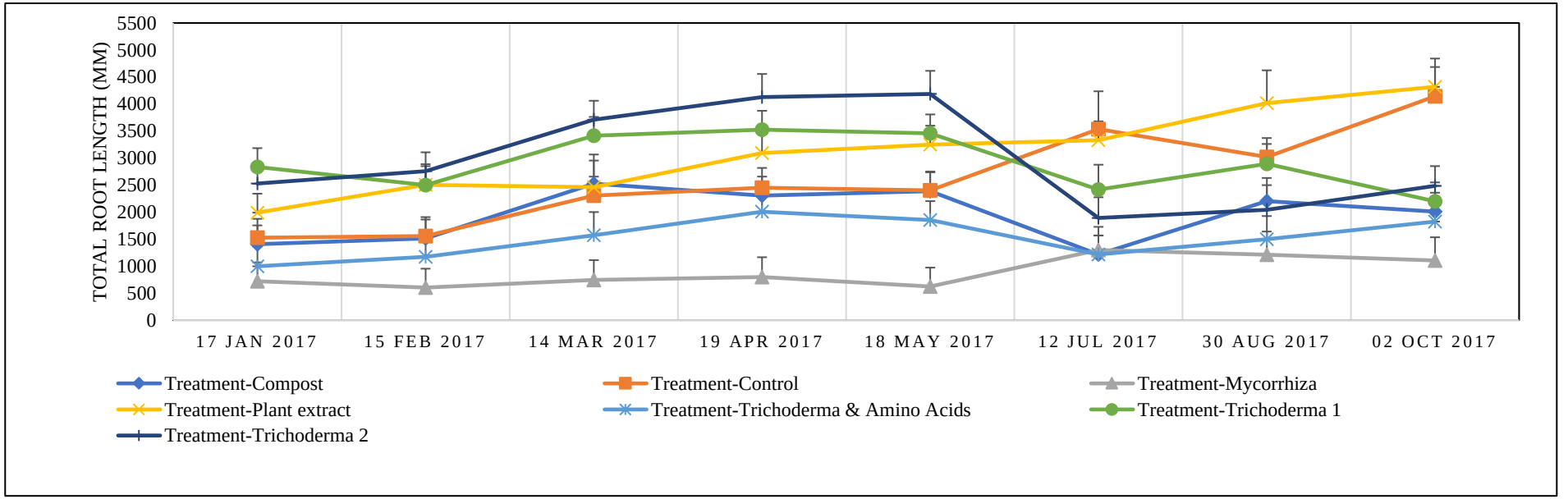
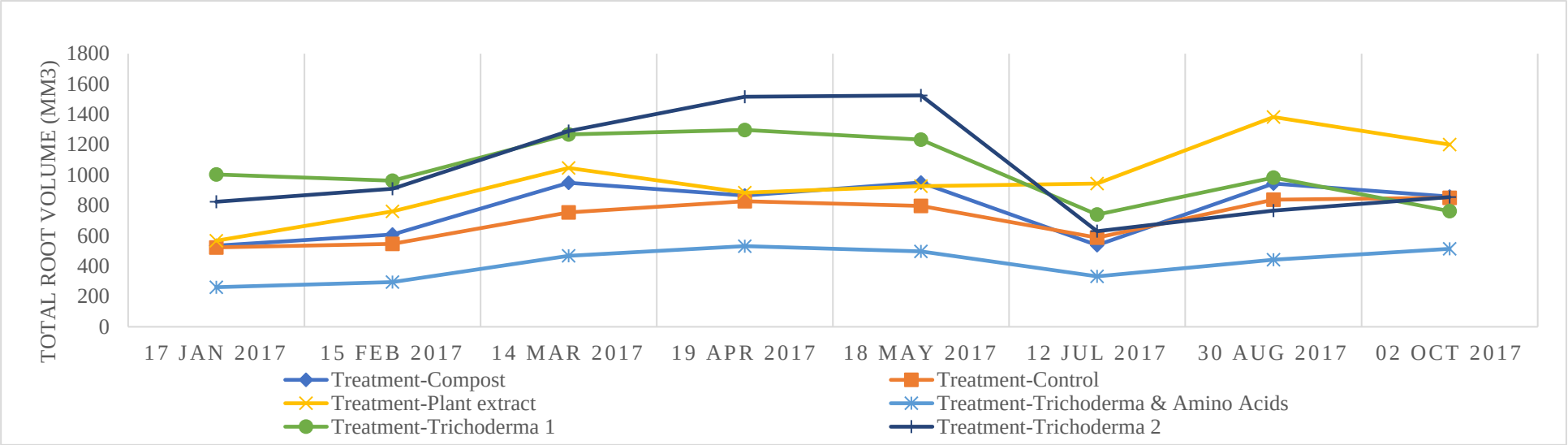


Figure 20: Average root count.100 cm^{-2} ± SE for roots with a diameter of >10 mm observed during destructive root analysis of young 'Granny Smith'/MM109 apple trees following the application of three biostimulant treatments (Compost, Plant Extract and Control) with establishment.



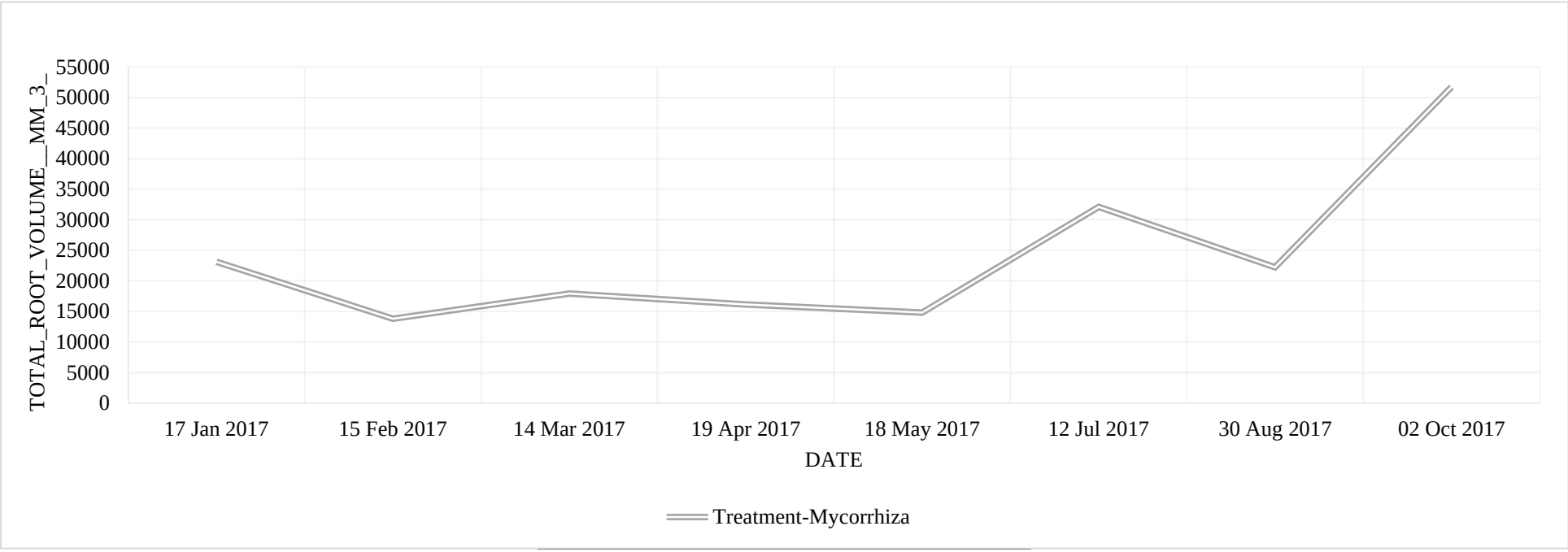
Date	Control	SE	Compost	SE	Mycorr	SE	Plant extract	SE	L - AA	SE	Trich 1	SE	Trich 2	SE
17-Jan-17	1526	350	1404	350	719	350	1991	350	997	365	2835	350	2527	350
15-Feb-17	1558	350	1513	350	603	350	2503	383	1173	350	2501	350	2758	350
14-Mar-17	2306	350	2526	428	746	365	2461	606	1573	428	3412	350	3711	350
19-Apr-17	2452	365	2307	350	799	365	3095	350	2006	383	3526	350	4129	428
18-May-17	2402	350	2387	350	623	350	3250	350	1853	350	3458	350	4187	428
12-Jul-17	3537	699	1216	350	1298	428	3331	350	1218	350	2419	458	1891	383
30-Aug-17	3021	350	2204	428	1211	428	4019	606	1498	428	2896	365	2041	458
02-Oct-17	4143	699	2010	350	1106	428	4320	365	1826	350	2200	350	2486	365

Figure 21: Seasonal trends for total root length of young ‘Granny Smith’/MM109 trees planted in fumigated soil, during the first season (Jan 2017 – Oct 2017), over all four soil levels (0 – 60 cm) and all treatments as well as the C control with supporting statistical analyses below.



Date	Control	SE	Compost	SE	Plant extract	SE	L - AA	SE	Trich 1	SE	Trich 2	SE
17-Jan-17	523.32	140.17	534.89	81.16	567.33	88.10	261.12	44.84	1004.00	237.25	823.43	119.82
15-Feb-17	546.12	148.82	608.69	74.88	760.63	106.37	295.34	43.12	962.61	201.57	909.15	120.46
14-Mar-17	754.55	170.90	949.45	129.27	1047.01	344.72	467.52	81.32	1267.30	222.28	1289.35	182.42
19-Apr-17	826.83	180.22	864.40	106.57	884.39	146.11	531.66	76.03	1297.59	226.73	1516.36	230.68
18-May-17	795.84	168.71	951.02	103.63	926.52	166.49	496.51	72.79	1232.81	252.61	1525.03	222.25
30-Aug-17	837.11	139.15	538.82	87.25	944.50	205.19	332.95	62.62	739.45	132.59	629.81	149.23
12-Jul-17	588.12	154.25	944.78	83.85	1382.41	530.77	441.65	94.96	983.46	146.56	765.42	132.09
02-Oct-17	850.54	220.21	858.11	102.97	1200.45	198.25	514.00	74.73	761.60	181.42	855.04	136.00

Figure 22: Seasonal trends for total root volume of young ‘Granny Smith’/MM109 trees planted in fumigated soil, during the first season (Jan 2017 – Oct 2017), over all four soil levels (0 – 60 cm) and all treatments as well as the Control, excluding Mycorrhiza treated trees, with supporting statistical analyses below.



Date	Mycorrhiza	SE
17-Jan-17	23106.33	2125.62
15-Feb-17	13805.89	2125.62
14-Mar-17	17941.41	2220.14
19-Apr-17	16134.83	2220.14
18-May-17	14803.56	2125.62
12-Jul-17	32117.54	2603.35
30-Aug-17	22239.93	2603.35
02-Oct-17	51740.03	2603.35

Figure 23: Seasonal trend for total root volume of young 'Granny Smit'/MM109 trees planted in fumigated soil, during the first season (Jan 2017 – Oct 2017), over all four soil levels (0 – 60 cm) for the Mycorrhiza treated trees, with supporting statistical analyses below.

PAPER 2: The Effect of Soil Applied Biostimulants on Microbial Colonization of Young Apple Tree Roots Planted in Fumigated Soil

ABSTRACT

Fumigation of soil infected with apple replant disease has become common practice to eradicate soil borne pathogens. Yet, increasing concerns regarding the effects thereof on beneficial soil microbial populations increasingly require a more environmentally friendly approach. The objective of this study was to investigate microbial colonization of roots indicated by the occurrence of *Trichoderma* spp. in fumigated soil, following the application of commercial soil biostimulants. Six biostimulant treatments and an untreated control were applied to 'Granny Smith'/MM109 trees at establishment of a commercial orchard, following soil fumigation with 1, 3-D-Chloropicrin: Compost, Plant extract, *Trichoderma* spp. (Trich 1, and 2), Plant extract, L-Amino Acids and Mycorrhiza. Root samples were collected and plated following treatment applications, where after visual scoring for the presence of *Trichoderma* spp., as a biological indicator for microorganism recovery, and *Sclerotia,* as a soil pathogen, were conducted. Within nine months after fumigation, both pathogenic and advantageous microorganisms populated the roots, but to various degrees, differing between treatments. The highest occurrence and growth of *Trichoderma* was found on roots from the Compost treatment. All treatments showed the presence of sclerotic bodies, with treatments Trich 2 and Plant Extract showing a significantly lower occurrence compared to the Control. Total root numbers (TRN) increased throughout the season in the top 30 cm soil depth, indicating consistent root recovery after fumigation in all treatments. Treatments Trich 1 and 2 stimulated the highest TRN and differed significantly from the other treatments on selected evaluation dates, although the rate of change after fumigation could not be quantified. Evidence from this study justifies future research into the recovery of the soil fertility and plant growth, based on biostimulant treatments following fumigation.

Key words: apple replant disease, compost, *Sclerotia*, soil recovery, *Trichoderma* spp.

1. INTRODUCTION

Pathogenic infection of the roots of young apple trees may occur in the nursery before planting or after establishment in existing commercial orchards, in case of diseased soil. Apple replant disease (ARD), which consist out of a complex of pathogenic organisms, is considered one of the most

devastating set-backs with orchard establishment, as it inevitably it leads to a loss of income with infected trees never reaching their full potential (van Schoor *et al.,* 2009). Some of the symptoms associated with ARD include reduced yield as result of stunted above- and below-ground growth, due to the loss of feeder roots associated with ARD. The severity of occurrence depends on many variables, which may include site-specific factors, micro-climate and soil structure (Rumberger *et al.*, 2007). As this complex of microbial pathogens varies from site to site, and may include the presence of nematodes, soil-borne fungi, bacteria and oomycetes (Rumberger *et al.,* 2007), it is difficult to exert full control with one approach or product. Nevertheless, in the past, fumigation with Methyl Bromide (MB) was considered very effective in controlling ARD, but in the process also eradicated most of both pathogenic and beneficial soil biota (Xie *et al.,* 2015).

Sclerotium-forming fungi is a major root pathogenic group responsible for significant economic losses in horticulture in South Africa, causing necrotic diseases such as white root rot on young apple trees (Lazarovits, 2001). The causal pathogen of white root rot of apple and pear trees in South Africa is *Rosellinia necratis*. Other necrotic plant pathogens such as *Sclerotium rolfsii*, *S. sclerotiorum* and *Rhizoctonia solani* are examples of sclerotium-producing fungi that are responsible for irreparable crop and financial losses on a global scale (Smith *et al.,* 2015).

In nurseries and commercial orchards, the destruction caused by white root rot is favoured by a combination of high temperatures and wet soils and is often associated with over irrigation in automated irrigation systems. *Rhizoctonia necratis* grows well in low acidic soils with pH5 to neutral soils, where the source of nutrition is largely provided by organic matter (Moorman and Daughtrey, 2002). The effective infection and spreading of many plant pathogenic organisms is highly dependent on the survival structures of the reproductive fruiting bodies formed by the pathogen (Smith *et al.,* 2015). In the case of white root rot, sclerotic-like masses form the gateway through which mycelial strands of *R. necratis* can infect the older plant tissues or the distal cells of the young roots (Moorman and Daughtrey, 2002). Survival structures such as spores and sclerotia (dense aggregation of tissue) are paramount for the fungi to endure environmental stresses such as low temperatures or freezing, or the absence of hosts, leaving the fungi exposed for microbial attacks to occur, resulting in the desiccation of the reproductive structures (Smith *et al.,* 2015). Persistence of sclerotia in the soil for up to several months is reliant on the production of biologically active secondary metabolites which may offer the fungi protection against a host of natural enemies during the dormant stages of its life cycle (Louw and Korsten, 2015; Smith *et al.,* 2015). The successful infection and evolution of *R. necratis* in young apple tree roots is associated with severe root damage, with yellowing and wilting of the basal leaves that are first observed, but ultimately resulting in the die-back of the young tree (Moorman and Daughtrey, 2002).

Fumigants are used widely as a preventative measure of control, together with the focussed deployment of antagonistic biological microorganisms to interfere with sclerotium formation (Lazarovits, 2001; Menge, 1982; Smith *et al.,* 2015). An unintended consequence with the use of effective fumigants is the devastating effect it generally has on all beneficial microorganisms inhabiting the same rhizosphere. The extent of the impact may differ between the selected fumigant and prevailing environmental factors and will determine the likelihood of which micro-organisms survive fumigation (Fang *et al.,* 2018a, 2018b; Sun *et al.,* 2020; Zhang *et al.,* 2019). Therefore, to restore a healthy soil environment after fumigation, additional interventions are required (Noling and Becker, 1994; Thakur *et al.,* 2018). In cases where soils are fallow, a cover crop can be planted to partially recover soil biology after fumigation (Shennan *et al.,* 2020; Vukicevich *et al.,* 2016). However, where faster rehabilitation is required, the application of compost and/or biostimulant products may be a viable consideration (Hellequin *et al.,* 2018; Zhen *et al.,* 2014).

One such biological microorganism that is suitable for control of plant pathogenic organisms, as well as alleviating plant stress and restoring balance in microbial populations within the soil, is *Trichoderma* (Naseby *et al.,* 2000). *Trichoderma spp.* are characterised by rapid growth in their natural environment (Schmoll and Schuster, 2010). In a field study of strawberry plants, the isolation of indigenous *Trichoderma* spp. showed more growth in fumigated than non-fumigated soil, or in soil with additional amendments such as compost (Leandro *et al.,* 2007). These highly adaptable robust fungi are known to secrete antibiotics and enzymes such as cellulase, which allows for metabolising of dead plant residues or cell walls, including, but not limited to, that of sclerotia forming soil pathogens (Harman *et al.,* 2008; Schmoll and Schuster, 2010), providing it with the ability of sustainable biocontrol and microbe growth regulation within production systems. Naseby *et al*. (2000) showed that *Trichoderma* spp. survived being autoclaved, where after it still had a promotive effect on the growth of young seedlings resulting in increased shoot and root weight. One such example is *Trichoderma asperellum* that reduces plant fatalities in onion caused by *Sclerotia cepivorum,* the causal pathogen of onion root rot (Rivera-Méndez *et al.,* 2020*)*. Similar results were also found in other vegetable crops such as tomato and beans (Amin *et al.,* 2010). Apart from being antagonistic to plant pathogens and inducing systemic plant defence responses, *Trichoderma* spp. contributes to plant health by altering the root growth of plants by influencing lateral root growth, as well as root hair formation (Contreras-Cornejo *et al.,* 2009).

The aim of this study was to quantify rehabilitation of soil microbial populations and accompanying root growth after fumigation and the application of biostimulant treatments. The objectives of this study were to investigate whether the presence of *Trichoderma* spp. on tree roots, known for its resilience to fumigation and beneficial effect on root growth, can i) be used as biological

indicator of soil biology recovery and ii), if soil microorganisms directly impacted on root growth dynamics which could mitigated transplant shock of 'Granny Smith' trees after fumigation.

2. MATERIALS AND METHODS

Experimental site

The experimental site was situated on a commercial apple farm, Lovenstein, in Vyeboom (34° 4' 55'' S; 10° 4' 12'' E), in the Western Cape, South Africa. The experiment was conducted on newly established 2-year-old 'Granny Smith' apple trees grafted on MM109 rootstocks, planted on 22 October 2016, at planting densities of 5 m x 2 m. Fumigation, using a combination of 1,3-D (490 $g.L^{-1}$) and CP (710 $g.L^{-1}$), was performed during September 2016, approximately seven weeks prior to planting, by a commercial company (BioScience Research, Cape Town, 7550), according to standard procedures.

Treatments

Seven treatments, including the untreated control which only received water after fumigation, were applied at planting, in October 2016, in a randomised complete design layout, with single trees (n=10) serving as experimental unit (Table 1). All protocols were followed according to the suppliers' recommendations using one of three application methods: i) application were either made directly to the planting hole prior to planting as was the case for the Compost treatment, ii) or by dipping the roots in the treatment solution immediately before planting, where after the soil was drench with water at planting or iii), treatments were applied as a soil-drench, near the roots of the tree following planting.

Soil amendments and management practices

Soil preparation was performed according to standard recommendations based on a chemical soil analysis. Plant rows were ridged (47 cm x 213 cm) to compensate for soil depth. Soils were supplemented pre-planting with the nutrient-based ameliorants Maxi Phos (Omnia Nutriology®) and KCl (Yara Africa Fertilizer Pty, Ltd., Paarl) on 20 May 2016 at 500 and 300 kg. ha^{-1} respectively. 'Enhancer' consisting of pelletised chicken manure (InteliChem Pty Ltd., Wellington) at 1.2 $kg.tree^{-1}$ was added at planting to enhance water holding capacity, improve soil organic content and stimulate microbial activity. 'Aldo' (unknown source), a controlled release NPK fertilizer, was applied two months after planting, at 200 $g.tree^{-1}$ in December 2016 and again, 13 months after planting, in November 2017. Compost-tea (Ecosoil, Grabouw) was applied at a rate of 240 $L.ha^{-1}$ (approximately 1000 trees) in monthly intervals from November 2016 to February 2017.

Micro-irrigation was installed 5 days after planting. Period prior to the installation of the irrigation system, irrigation of trees was conducted manually at approximately 15-25 L per tree per day. Initially the micro-irrigation schedule provided irrigation for eight hours, once a week, for the first month after planting. In 2016/17, from December to February and again from May to November 2017, trees were irrigated for five hours, every 6th day. During March and April, trees were irrigated for an additional hour, with every second cycle. In 2017/18, trees were irrigated every 5th day for four hours and again for an additional two hours, during March and April, with every second cycle.

Sampling and data collection for microbial colonization

Approximately 200 g of roots from each treatment (n=5) were harvested using secateurs, on 12 July 2017, nine months after planting, at a depth of 20-30 cm, depending on the natural distribution of the roots. All roots were harvested on the same side of the tree, where after it was placed into plastic bags and kept at 4 ℃ for four days prior to plating onto an agar growth medium consisting of out 39 g of potato dextrose agar (PDA) diluted in one litre of deionised water. After being autoclaved for 45 minutes (200 ℃, 2 kPa), the growth medium was cooled to approximately 38 ℃, before adding 0.04 g of streptomycin per litre of growing medium to inhibit any bacterial growth. Using a laminar flow bench, the medium was poured into a 90 mm petri-dishes to cover 1.5 mm of the surface area, where after the plates were cooled before being stored at 4 ℃ until further use. Ten plates were allocated per replicate.

The first 11 samples of tree roots were plated out on 18 July 20017. After two days of incubation, the first visible fungal growth was observed. On 20 July 2017, the remaining 12-35 samples were plated, where after observations were carried out at room temperature (25 ℃), on two, four and eight days following the incubation period. Visual identification of *Trichoderma* spp. on PDA growth medium was assessed based on two classes of infection: less than 50% of *Trichoderma* spp. growth (Fig. 1A), or more than 50 % of *Trichoderma* spp. growth (Fig. 1B). In addition, a visual evaluation for the presence of *Sclerotia* was performed on the same samples, where the incidence of sclerotic bodies was noted categorically, as either present or absent (Fig. 1C), with results expressed as percentage per replicate.

Sampling and data collection for root growth dynamics

Plant performance was determined, approximately every four to six weeks during the season, as total root number (TRN), using a root scanner (CID -600) and minirhizotron. Three minirhizotron tubes were installed for each treatment, according to a total randomized design (Paper 1). Digital images of the roots were collected with a CI-600 *In Situ* Root Imager (CID Bio-135 Science in Camas, WA, USA) for the two soil depths (0-15 cm; 15-30 cm) relevant to root colonisation by *Trichoderma* spp.

Root numbers were calculated with the RootSnap! image analysis software (CI-690, Version 1.3.2.25, CID Bio-Science Inc., 143 Camas, WA, USA.).

Statistical Analysis

Statistical analyses were performed on the average percentages, using a Two-way analysis of variance (ANOVA), generalized linear model (GLM) procedure in SAS 9.4 (SAS Institute Inc. 2004, Cary, USA). Means were separated by Least Significant Differences (LSD) when significant differences occurred at a 5% confidence level ($p \leq 0.05$). In addition, regression analyses were performed, per tube, representing seasonal trends of the various treatments over time. This was presented with confidence levels and SE values using XLSTAT (XLSTAT statistical and data analysis solution. New York, USA. https://www.xlstat.com)

3. RESULTS

Microbial colonisation

Trichoderma spp. were present in all treatments, approximately nine months after treatment. Treatments all differed significantly from one another, with respect to the samples observed showing < 50 % growth of *Trichoderma* spp. (Table 2). The incidence of detection differed significantly between treatments, with treatment LAA showing the lowest *Trichoderma* spp. growth where 42% of all samples had <50% colonization on tree roots. This was followed by Trich 1, Control, Plant extract, Mycorrhiza, Trich 2 and Compost, but where *Trichoderma* spp. root colonization was the highest with only 20 % of the samples that had < 50% colonization on tree roots (Table 2).

Presence of Sclerotia *spp.*

The presence of *Sclerotia* differed significantly between four of the seven treatments (Table 1). The highest occurrence of *Sclerotic* bodies was reported in the Control (60 %) and Mycorrhiza (52 %) treatments. However, these treatments did not differ significantly from Trich 1 (32 %), LAA (42 %) and Compost (28 %). The lowest occurrence of sclerotic bodies was found in the Trich 2 (20 %) and Plant Extract (24 %) treatments, which differed significantly from the Control and Mycorrhiza treatments at the 5 % confidence level (Table 1).

Root growth dynamics

Total root number (TRN) differed significantly between treatments on every evaluation date in levels 3 and 4, for all dates except February and March 2017.

Level 3: Trich 1 had significantly higher TRN than all treatments in December 2016. In January 2017, TRN of Trich 1 was significantly higher than LAA, Mycorrhiza and Compost, but other treatments did not differ significantly. In February 2017, a significantly higher TRN was observed in treatments Trich 1 and 2, Plant Extract and the Control than in Mycorrhiza, but the rest of the treatments did not differ significantly. In March 2017, TRN was significantly higher in Trich 1 than LAA, Mycorrhizae and Compost, with other treatments not differing. In April and May 2017, a significantly higher TRN was recorded in treatments Trich 1 and 2 than the Compost, Mycorrhizae and LAA treatments, with the rest not differing significantly (Table 3). In July 2017, TRN was significantly higher in the Plant Extract treatment than the Compost, Mycorrhizae, LAA and Trich 2 treatments, with the rest not differing significantly (Table 3). Finally, in August 2017, the highest TRN were reported in the Control and Plant Extract treatments, that differed significantly only from the Compost, Mycorrhizae and LAA treatments.

Level 4: In December 2016 and January 2017, TRN was significantly higher in the Control than all treatments, except Trich 1 and 2. In April and May 2017, a significantly higher TRN was reported in Trich 1 and 2 than all treatments, but not for the Control. In July 2017, the Control had significantly higher TRN than all treatments except Trich 1 and 2. During August 2017, the Control, Plant Extract and Trich 1 treatments had a significantly higher TRN than the rest of the treatments.

Treatment effects varied amongst dates and therefore trends were followed to indicate possible treatment effects. An overall increasing trend in TRN across treatments was observed from the topsoil level 0-15cm (level 4) (Fig. 2) towards 15-30 cm (level 3) (Fig. 3). The Plant Extract treatment showed a greater decrease in TRN from level 3 to level 4 compared to the Trich 1, Trich 2 treatments and the Control (Fig. 2, Table 3). A steady increase in TRN in the Control was noticed from December 2016 to August 2017, for both soil depths (Fig. 2, 3).

The Mycorrhizae treatment displayed the lowest TRN in level 3, with an alternating pattern during the season (Fig. 3). The Mycorrhizae treatment displayed an increase in TRN towards April, followed by a decline in May and an increase from June to August. TRN in the Plant Extract treatment followed a similar trend to the Control, but a decrease in TRN February and July 2017.

For treatment LAA, an increase in TRN was reported in level 3 from December 2016 to March 2017, followed by a decline from April 2017 – July 2017, with another increase in August 2017 (Fig. 3). A similar trend was observed in level 4, except for a decline in TRN from May – July 2017 (Fig. 2).

Treatment Trich 1 showed the highest TRN in level 3, although this was not significantly different from the Control (Table 3).

A similar trend for TRN was noticed in treatments Trich 1 and Mycorrhizae, with several fluctuations between December 2016 and August 2017 (Fig. 2, 3). In treatment Trich 1 level 4, a

continuous increase in TRN occurred from December 2016 – May 2017, followed by a decline in July 2017 and then an increase in August 2017, which was not significantly different from the Control (Fig. 2). TRN for treatment Trich 2 showed a general increase over time, with a decrease in TRN in July 2017 in level 3, and for February, May and July 2017 in level 4 (Fig. 2, 3).

4. DISCUSSION

Microbial colonisation

Trichoderma spp.

Trichoderma spp. colonization was recorded for roots of all treatments, nine months after application, irrespective of whether the specific treatments contained formulated or natural sources of *Trichoderma* spp. It is very likely that natural occurring species of *Trichoderma* were present in the soil, which would partly explain why *Trichoderma* spp. were observed in all treatments at evaluation. However, this is only speculation and was not quantified in the scope of the present study. Nevertheless, although the findings of De Los Santos *et al*. (2003) showed that the presence of wild *Trichoderma* spp. increased after fumigation with chloropicrin. In addition, Naseby *et al*. (2000) confirmed the robust nature of *Trichoderma* spp. as it was able to withstand the high temperature and pressures conditions prevalent during autoclaving.

Treatments which displayed the highest *Trichoderma* spp. root colonization were Mycorrhiza, Compost and Trich 2, which is unexpected, since the inclusion of *Trichoderma* in the treatments differed from i) no *Trichoderma* species included (Mycorrhiza), to ii) possibly have *Trichoderma* spp. included (Compost), to iii) having four confirmed *Trichoderma* spp. included respectively (Table 2). Poveda *et al.* (2019) also showed that the simultaneous application of *Trichoderma harzianum* and arbuscular mycorrhizal fungi (AMF) significantly increased the colonization of *Trichoderma* and the presence of AMF in the roots of *Arabidopsis* and rapeseed plants. This was related to a phytohormone interaction due to the induced systemic plant response after infection of AMF occurred in the plant roots (Poveda *et al.*, 2019). This could provide a possible explanation for the high *Trichoderma* colonization observed with the Mycorrhiza treatment; however, quantification of this scenario was beyond the scope of this study and could therefore only be seen as speculation.

Compost amendments alter the soil microbial composition when applied to ARD infected soils (Yao *et al.*, 2006), as well as fumigated soil (Leandro *et al.*, 2006). The use of compost and *Trichoderma hamatum* together, in field conditions, showed that compost promoted the survival of *Trichoderma* in soil and manipulated soil microbial populations of indigenous *Trichoderma* spp. on the roots of strawberry plants (Leandro *et al.*, 2006). Compost provided a suitable organic substrate for *Trichoderma* (Krause *et al.*, 2007) and could be a possible explanation for the high *Trichoderma* root colonization observed during our study, however this should be confirmed by future research.

Different modes-of-action are exerted by the respective treatments, but offers no conclusive explanation for the observed higher *Trichoderma* spp. root colonization incidence reported for all these very different biostimulant treatments

Sclerotia forming spp.

The presence of sclerotic bodies nine months after fumigation of the orchard is concurrent with findings showing that pathogens form robust fruiting structures to enhance their chance of survival (Bolton *et al.*, 2006). Smoli and Kowalska (2018) reported that sclerotic fruiting bodies to be chemically resistant, whereas Pecina *et al*. (2016) showed that, despite the detrimental effect chloropicrin application may have on phytopathogenic organisms, complete eradication of soil borne pathogens was not achieved. It is thus speculated that the fumigation of soils in our trial was not successful to eliminate all soil borne pathogens for a period of up to nine months following fumigation.

The occurrence of sclerotic fruiting bodies was the highest in the untreated Control (60 %), followed by the Mycorrhiza (52 %) and Trich AA (42 %) treatments (Table 2). In addition, the Control and Trich AA also had the lowest *Trichoderma* spp. root colonization. This indicated that the remainder of the treatments applied with direct *Trichoderma* isolates displayed a form of potential biocontrol by *Trichoderma spp.* over sclerotia fruiting bodies isolated in this study for these treatments. This is concurrent with the findings of Rivera-Méndez *et al*. (2020) who confirmed significant disease control on onions under tropical climatic conditions. This was confirmed on several other crop types like tomatoes (Clarkson *et al.*, 2004), cotton and beans (Elad, 1980) with *Trichoderma* isolates. *Trichoderma spp.* also promoted crop growth in the absence of *Pythium* and reduced plant damage caused by *Pythium* (Naseby *et al.*, 2000). It is speculated that natural occurring *Trichoderma* could have contributed to soil recovery, similar to reported in the study of De Los Santos *et al*. (2003). This would offer some explanation why the untreated Control showed comparable results than that obtained for treatments that had specific *Trichoderma* included in their formulation.

Root growth dynamics

The lower TRN values reported in the topsoil layer (level 4) compared to the subsoil level (level 3) for most dates, indicates a less favourable root growth environment in the topsoil often reported as result of high soil temperatures and evaporation rates from soil in the rhizosphere of young, established apple orchards (Nicholson, 2012). Significant differences between treatments for TRN were recorded throughout the season, but varied according to evaluation date (Table 3). Yet, in general the effect of the treatments on TRN varied according to either the composition or the mode-of-action of the various products (Fig 2, 3).

Mycorrhiza consistently showed considerably lower TRN than the other treatments (Table 3). In addition, the TRN did not increase as substantially as for the other treatments during the season (Fig 2, 3). This confirms results reported in Paper 1, where it was concluded that Mycorrhiza does not increase root numbers during colonization, but is associated more with an increase in root area. For Trich 1 and 2, TRN increased from Dec to July, before declining towards Aug (Table 3). TRN for these treatments were often significantly higher than for Mycorrhiza, but did not always differ significantly from the other treatments (Table 3). TRN of LAA and Plant Extract treatments and the Control ranged between those reported for Mycorrhiza, Trich 1 and Trich 2 (Table 3). Seasonal trends showed an increase in TRN for the Control, LAA, Plant Extract and Trich 2, in both levels, and a declining trend for Trich 1, in both levels (Fig 2, 3). Compost showed an increasing trend in level 3, but decreasing trend in level 4 (Fig 2,3). Mycorrhiza showed an increasing TRG in level 3, but a declining trend in level 4 (Fig 2, 3). TRN trends for Mycorrhiza were similar in both levels and indicated minimum increases during the season.

The exact role, species of *Trichoderma spp.* and the influence thereof on the root dynamics was outside the scope of the study. Yet, of interest is that TRN increases in some of our biostimulant treatments are concurrent to findings by Contreras-Cornejo *et al*. (2009), where the addition of *Trichoderma atroviride* induced a plant systemic response, which lead to the increase of the plant phytohormone auxin, which influenced root growth. Yet, as this is not the case for all *Trichoderma* spp., if the aim is to stimulate root growth, products containing specific species should be selected. In our study, the value of providing additional amino acids for *Trichoderma* colonisation in the biostimulant formulation could not be demonstrated and requires further research. The general increase in TRN towards the end of season compared to establishment confirms root growth dynamics of young, non-bearing apple trees (Van Zyl, 2016; Lötze *et al.*, 2018). The diverse seasonal TRN trends for the treatments indicated the role of the specific biostimulant group with regard to expression of the biostimulant reaction alluded to by Rouphael *et al*. (2020). The direct contribution of *Trichoderma* spp. colonisation over this period towards TRN was not quantified in this study, but may present an interesting future investigation. Competition for assimilates between the roots and shoots with the progression of the season may also have contributed towards the TRN trends, but was not quantified in this paper.

5. CONCLUSION

The objective of this study was to investigate whether the presence of *Trichoderma* spp. on tree roots can be used as a biological indicator of soil biology and tree root recovery, nine months after fumigation, following the application of a range of commercial biostimulants. *Trichoderma* spp. is

speculated to be central to altering signalling pathways between the plant, soil pathogens (scletoria) (Vinale *et al.*, 2008; Rivera-Méndez *et al.*, 2020).

The most prominent finding that emerged from this study is that nine months after fumigation, pathogenic *Sclerotia* fruiting bodies and advantageous microorganisms (*Trichoderma* spp.) were present in substantial amounts, in all treatments. This provides evidence of a sequential recovery of soil microbial life after fumigation. The Control treatment hosted the highest presence of sclerotia bodies. Yet, it displayed comparable incidences of *Trichoderma* infection to that of treatments, where *Trichoderma* was included in the formulation, suggesting possible colonisation of natural species that survived fumigation. These results emphasised the complexity of soil microbial life after fumigation, particular with regard to the role of natural surviving microorganisms, the availability of food sources that regulates competition between organisms, and the impact of the initial infection of nursery tree roots with sclerotia before planting, all factors that may impact soil biota recovery following fumigation. In addition, compost and Trich 2 treatments showed potential antagonism and thus biocontrol against sclerotia bodies via *Trichoderma* spp. This finding warrants further research following standard protocols suitable for pathological investigations.

Seasonal trends for TRN in both levels suggested a consistent increase in TRN from establishment until 9 months thereafter, for most treatments except Trich 1. Treatments differed significicantly with regards to the slope of the increase. This implicated positive root growth in terms of TRN in the majority of the treatments, with limited or absence of severe stress on the below ground tree components during the first season after fumigation. However, it is unknown whether more frequent applications of the biostimulant treatments would have facilitated higher *Trichoderma* spp. colonisation resulting in in increasing root number than a single application, and may have been more effective in reducing transplant shock and increase plant performance after establishment in a fumigated soil.

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7. TABLES AND FIGURES

Table 1: Treatment details for the 'Granny Smith'/MM109 apple trial on Lovenstein farm, Vyeboom, for biostimulant applications during establishment in October 2016, after soil fumigation.

Treatment	Product/ Supplier	Application method
Control (water) (Fumigation only)	Control	-Add water to the tree at planting
Compost Cow manure and garden refuge	Stellenbosch University	-Apply in the planting hole at planting
Mycorrhiza	Mycogel from Kimitec/ Wenkem SA (www.wenkem.co.za)	-0.5 ml of product per tree, applied at planting or within 3-4 days of planting -No phosphates or pesticides should be applied within 14 days after application, only use of organic fertilizer
Plant Extract Plant extracts, PGPB - Bacillus Bacteria (95%), microbes & naturally occurring organic-soluble humates	Aqua Clean SA Sludge Abate Blue Planet SA (www.blueplanet-sa.com)	-150 ml of well-mixed product added near tree roots -Applied with enough water to spread the product
Trichoderma 1 (Trich 1) Contain 8 different isolates of Trichoderma: -*T.asperellum* (3) -*T. hamatum* (1) -*T. atroviride* (2) -*T.harzianum* (2)	Bio-Tricho Liquid Agro-Organics (www.agro-organics.co.za)	-Submerge tree roots should in a solution of spore suspension of 400 ml.100L^{-1} product in water -Soak for 10 minutes or longer before transplanting -Commercial soil application at 1L. ha^{-1}
L-Amino Acids (LAA) Contains 19 synthesized L-amino acids, oligo-peptides and nutrients	Aminostim from Bioscience Research (www.brsa.com)	-A liquid formulation mixed with water & applied by means of foliar spray at 0.5L– 1L. ha-1), -Drip irrigation at 1.5L to 3L. ha-1 or -Apply in hydroponics at lower rates to young plants and during flowering stages -Applied commercially as a liquid through irrigation every 4 weeks at 25ml per tree or 15L.ha-1
Trichoderma 2 (Trich 2) Contains 4 selected species of *Trichoderma* endophytes	Excalibur Gold ABM Africa (www.abm1st.com)	-Submerged trees into a solution of the product or as a drench (500 g.ha^{-1})

Table 2: Incidences of Trichoderma growth less than 50% and the incidence of *the presence of sclerotia. (Indicating the presence of Sclerotia spp.)* observed on roots of the 'Granny Smith'/MM109 trees in July 2017, approximately 9 months following establishment and biostimulant treatment on fumigated soil of an apple orchard in Lovenstein, Vyeboom in September 2016.

Treatment	Incidence of Trichoderma growth < 50%	Sclerotia incidence
Control	34 [c]	60 [ab]
Mycorrhiza	28 [e]	56 [ab]
Trichoderma 1 (Trich 1)	38 [b]	32 [abc]
Trichoderma 2 (Trich 2)	24 [f]	20 [c]
Compost	20 [g]	28 [bc]
L-Amino Acids (LAA)	42 [a]	42 [abc]
Plant extract	30 [d]	24 [c]
P (<0.05)	**<.0001**	**0.0416**

Table 3: The total root number for 'Granny Smith'/MM109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap! software for the biostimulant treatments at two soil depths (Level 3 (15-30 cm) and 4 (0 – 15 cm)) below the soil surface during 2016/17.

Total Root Number, Season 1 (201617)									
Level /Soil depth	**Treatment**	**15 December 2016**	**17 January 2017**	**15 February 2017**	**14 March 2017**	**19 April 2017**	**18 May 2017**	**12 July 2017**	**30 August 2017**
3 (15-30 cm)	Control	34.7 b	59.3 ab	67.3 ab	74.7 abc	77.3 ab	81.7 ab	84.3 ab	98.7 a
	Compost	21.3 b	29.0 bc	34.3 bc	39.7 bcd	41.3 bc	46.0 bc	29.0 bcd	39.7 bc
	Mycorrhiza	9.0 b	12.0 c	9.3 c	10.0 d	8.3 c	7.0 c	15.7 d	16.3 c
	Plant extract	29.3 b	49.3 abc	57.3 ab	69.0 abc	75.0 ab	84.0 ab	90.3 a	98.7 a
	L-Amino Acids (LAA)	6.3 b	20.3 bc	24.7 bc	30.7 cd	26.5 c	23.3 c	17.3 cd	24.7 c
	Trichoderma 1	86.3 a	89.0 a	85.7 ab	106.3 a	104.0 a	106.7 a	74.0 abc	85.7 ab
	Trichoderma 2	40.7 b	58.3 ab	64.3 ab	79.3 ab	104.5 a	106.5 a	49.7 bcd	64.7 abc
	P-Value	**0.0068**	**0.0301**	**0.0335**	**0.0067**	**0.0029**	**0.0019**	**0.0470**	**0.0131**
4 (0-15cm)	Control	35.3 a	44.3 a	32.7 ns	48.0 ns	44.5 ab	55.7 a	57.3 a	66.3 a
	Compost	6.3 c	13.7 cd	16.0	21.3	22.0 b	22.3 b	9.3 c	14.3 b
	Mycorrhiza	6.0 c	8.3 d	9.3	9.3	13.3 b	7.7 b	10.7 bc	15.7 b
	Plant extract	9.0 bc	21.3 bcd	9.0	14.3	19.7 b	21.0 b	12.7 bc	31.0 ab
	L-Amino Acids (LAA)	2.3 c	8.7 d	10.0	14.0	15.5 b	13.3 b	9.0 c	13.0 b
	Trichoderma 1	27.7 ab	42.7 ab	45.7	54.7	59.7 a	60.7 a	46.0 ab	60.7 a
	Trichoderma 2	21.0 abc	31.7 abc	30.7	50.7	75.0 a	74.5 a	27.0 abc	42.0 b
	P-Value	**0.0198**	**0.0101**	**0.0836**	**0.0663**	**0.0124**	**0.0038**	**0.0388**	**0.0262**

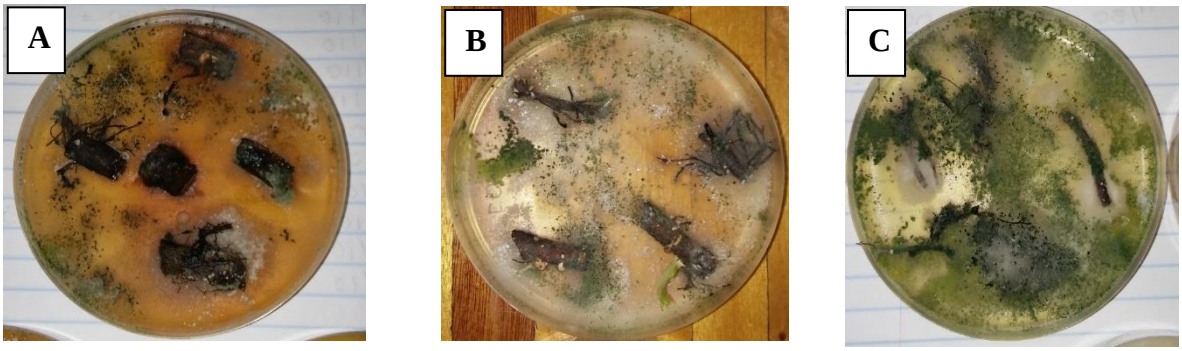
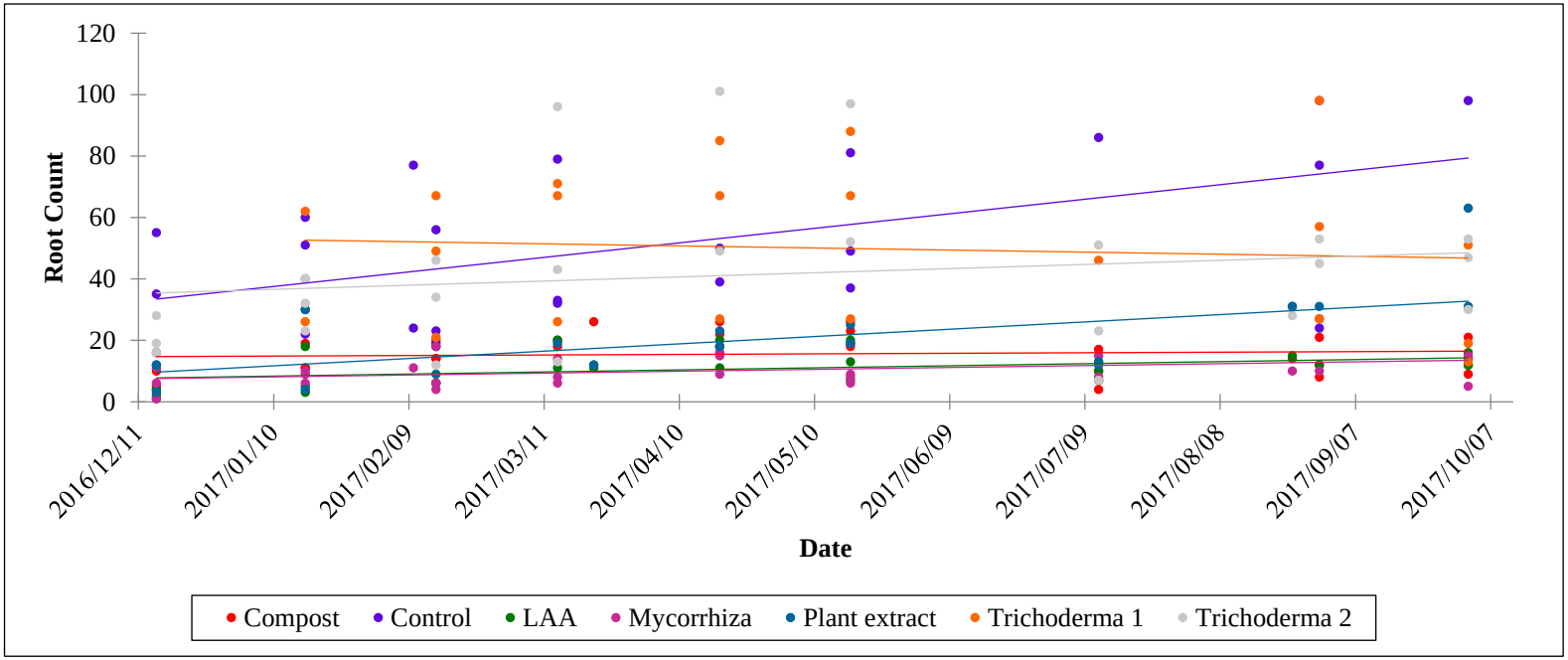
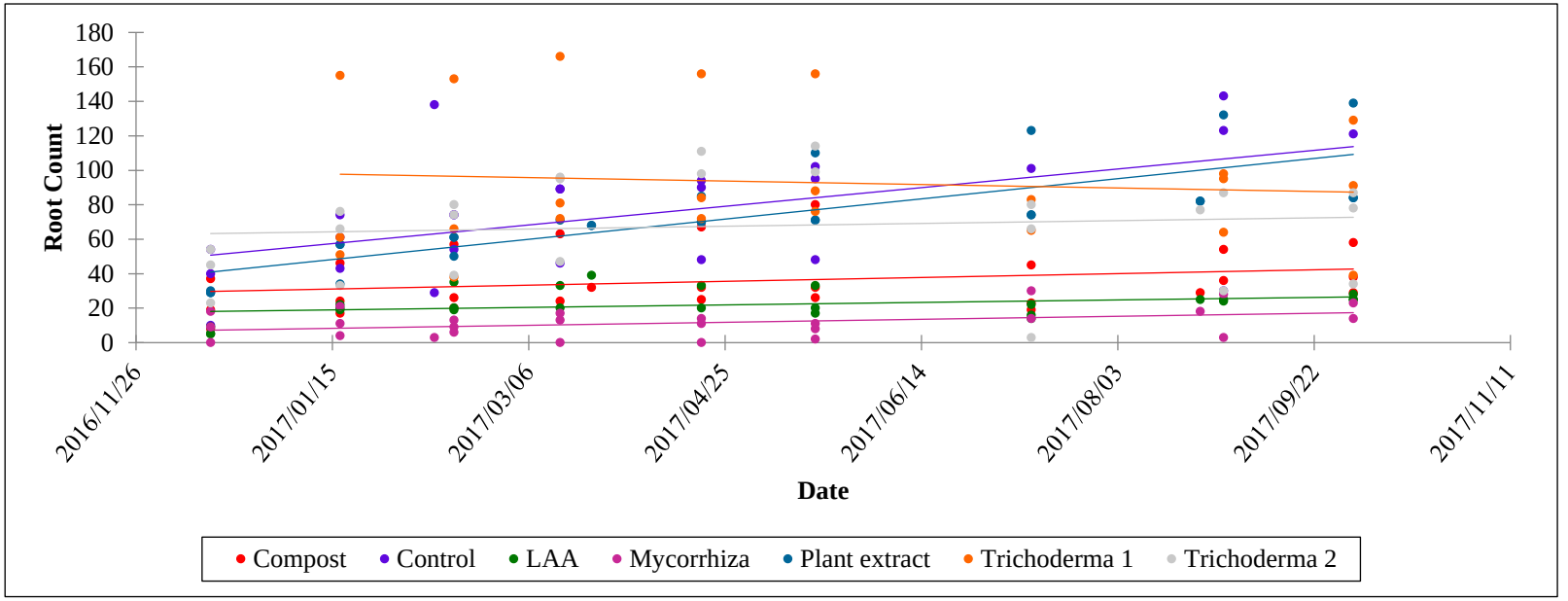


Figure 1: Visual representation of *Trichoderma* spp. growth (A) of less than 50%, (B) growth of more than 50%; (C) showing the presence of *Sclerotic* spp., indicated by large black circular fruiting bodies.



Date	Control	SE	Compost	SE	LAA	SE	Mycorrhiza	SE	Plant extract	SE	Trich 1	SE	Trich 2	SE
15-Dec-16	35.33	9.8	6.33	9.8	2.33	9.8	6.00	9.8	9.00	9.8			21.00	9.8
17-Jan-17	44.33	9.8	13.67	9.8	8.67	9.8	8.33	9.8	21.33	9.8	42.67	9.8	31.67	9.8
10-Feb-17	50.50	12.1					11.00	17.0						
15-Feb-17	32.67	9.8	16.00	9.8	10.00	9.8			9.00	17.0	45.67	9.8	30.67	9.8
14-Mar-17	48.00	9.8	19.00	12.1	15.50	12.1	9.33	9.8	19.00	17.0	54.67	9.8	50.67	9.8
22-Mar-17			26.00	17.0	11.00	17.0	9.33	9.8	12.00	12.1				
19-Apr-17	44.50	12.1	22.00	9.8	15.50	12.1	13.33	9.8	19.67	9.8	59.67	9.8	75.00	12.1
18-May-17	55.67	9.8	22.33	9.8	13.33	9.8	7.67	9.8	21.00	9.8	60.67	9.8	74.50	12.1
12-Jul-17	86.00	17.0	9.33	9.8	9.00	9.8	11.50	12.1	12.67	9.8	46.00	17.0	27.00	9.8
24-Aug-17			14.00	17.0	15.00	17.0	10.00	17.0	31.00	12.1			28.00	17.0
30-Aug-17	66.33	9.8	14.50	12.0	12.00	12.1	18.50	12.1	31.00	17.0	60.67	9.8	49.00	12.1
02-Oct-17	98.00	17.0	14.67	9.8	13.33	9.8	10.00	12.1	47.00	12.1	27.67	9.8	43.33	9.8

Figure 2: Seasonal trends for total root number of young 'Granny Smiht'/MM109 trees during the first season (December 2016 – December 2017) for level 4 (0-15 cm soil depth) with supporting statistical analyses below.



Date	Control	SE	Compost	SE	LAA	SE	Mycorrhiza	SE	Plant extract	SE	Trich 1	SE	Trich 2	SE
15-Dec-16	34.67	15.30	21.33	15.30	6.33	15.30	9.00	15.30	29.33	15.30			40.67	15.30
17-Jan-17	59.33	15.30	29.00	15.30	20.33	15.30	12.00	15.30	49.33	15.30	89.00	15.30	58.33	15.30
10-Feb-17	83.50	18.74					3.00	26.50						
15-Feb-17	67.33	15.30	34.33	15.30	24.67	15.30	9.33	15.30	57.33	15.30	85.67	15.30	64.33	15.30
14-Mar-17	74.67	15.30	43.50	18.74	26.50	18.74	10.00	15.30	71.00	26.50	106.33	15.30	79.33	15.30
22-Mar-17			32.00	26.50	39.00	26.50			68.00	18.74				
19-Apr-17	77.33	15.30	41.33	15.30	26.50	18.74	8.33	15.30	75.00	15.30	104.00	15.30	104.50	18.74
18-May-17	81.67	15.30	46.00	15.30	23.33	15.30	7.00	15.30	84.00	15.30	106.67	15.30	106.50	18.74
12-Jul-17	101.00	26.50	29.00	15.30	17.33	15.30	22.00	18.74	90.33	15.30	74.00	18.74	49.67	15.30
24-Aug-17			29.00	26.50	25.00	26.50	18.00	26.50	82.00	18.74			77.00	26.50
30-Aug-17	98.67	15.30	45.00	18.74	24.50	18.74	15.50	18.74	132.00	26.50	85.67	15.30	58.50	18.74
02-Oct-17	121.00	26.50	41.67	15.30	26.00	15.30	18.50	18.74	102.33	15.30	86.33	15.30	66.33	15.30

Figure 3: Seasonal trends in the total root number of young 'Granny Smith'/MM109 trees during the first season (December 2016 – December 2017) for level 3 (15-30 cm soil depth) with supporting statistical analyses below (Table 5)

PAPER 3: The Effect of Biostimulants at Establishment on Vegetative Parameters of Young, Non-bearing Apple Trees after Fumigation

ABSTRACT

Establishing new apple orchards on soils previously cultivated with apples renders the new plantings susceptible to apple replant disease (ARD). Although fumigation is an effective strategy to combat ARD, it is also detrimental to naturally occurring soil microbes and promotes the degradation of soil fertility. Several biostimulant products have been formulated to contain microorganisms and/or substances which, if applied after fumigation, may mitigate the recovery of the soil microbial life and assist in transplant shock, while promoting initial plant growth. This study examined the effect of commercial biostimulants on the vegetative above ground growth of young 'Granny Smith'/ MM109 apple trees that were established in fumigated ARD soil. Growth was quantified over two consecutive seasons following planting by: recording stem water potential; photosynthetic capacity; stomatal conductance; leaf nutrient composition; leaf total soluble solids; stem diameter and determining the length of one-year-old shoot growth. No significant differences between treatments regarding vegetative aerial growth were found, during either of the seasons, regardless of the type of stress mediation. This confirmed existing reports, indicating that a longer period is required to evaluate the effects of biostimulants on perennial crops under field conditions. In addition, a severe drought that prevailed during establishment could have compromised the performance of the biostimulant products regarding above ground growth, as significant treatment effects were reported for root growth (Paper 1) and soil recovery (Paper 3) in the same study. As treatments in this study were only applied once, at planting, adjustment in the application protocol should evaluated in future studies as it may result in the short-term expression of treatment effects in above ground growth performance, which will aid in a faster assessment of the efficacy of biostimulant treatments to ameliorate the effect of fumigation on plant production.

Key words: *Granny Smith; microorganisms; root-shoot competition; stem water potential; transplant shock*

1. INTRODUCTION

A priority after planting an apple orchard is to fill the allotted space with bearing wood (Miller, 1983). Any practices or environmental conditions that negatively impacts tree survival and growth would increase the time to full production capacity and thus reduce profitability of the orchard.

When apple trees are transplanted from the nursery into a newly established orchard, overcoming transplant shock and establishing a robust root system for subsequent tree growth is important to ensure above ground growth. Transplant shock is generally defined as the stress caused by removing most of the apple tree roots when transferring the tree from the nursery, often with the roots exposed, to its new environment (Harris and Bassuk, 1993). With the excavation and transport of the young trees from the nursery to the orchard, the finer roots and root tips are often damaged or lost, resulting in the absorption of nutrients being greatly reduced. Thus, with establishment in the orchard, the young tree is challenged with adapting to its new environmental conditions, in addition to having a compromised root system. Stunted growth or die-back of the growth tip (Tewoldemedhin *et al.,* 2011a; Van Schoor *et al.,* 2008) which limits potential growth and yield of the newly established apple trees is therefore most often associated with transplant stress, apple replant disease (ARD), various environmental strains and poor soil conditions (Tewoldemedhin *et al.,* 2011).

ARD is caused by a complex of soil pathogens that occur on sites that were previously planted with apples (Tewoldemedhin *et al.,* 2011; Van Schoor *et al.,* 2009). As this condition results in the setback of young tree growth and often, death, it is vital to address this disease before replanting an existing orchard, when there is any suspicion of possible ARD. Although ARD under South African conditions is more linked to biological factors and fungal complexes in soils, specific abiotic factors may also affect the expression of stress symptoms by the tree.

Until recently, ARD was successfully addressed by soil fumigation with methyl bromide (MB) as the norm for the South African deciduous fruit industry (personal communication, C. Stanton, Draslovka Services, Stellenbosch). However, the banning and complete removal of MB in developing countries by 2015 (Ajwa *et al.,* 2010; Tripp, 1988), along with calls for more environmentally sustainable agriculture practices, sparked the search for alternative approaches (Ajwa *et al.,* 2010; Guo *et al.,* 2018; Lazarovits, 2001; Leinfelder and Merwin, 2006; Mazzola and Mullinix, 2005; Porras *et al.,* 2007; Van Schoor *et al.,* 2009). One such alternative fumigant that has been considered is 1, 3-D-Chloropicrin, in addition to practices that contain less or no chemicals such as using *Brassica napus* seed meal in combination with cover crops or with

post-planting mefenoxam soil drenches (Mazzola and Mullinix, 2005). Yet, due to site-specific variables in orchards (Tewoldemedhin *et al.,* 2011), these alternative approaches have not yet attained the same level of efficiency as MB in controlling ARD. Thus, no universal remedy is yet available adopt commercially. This calls for research to develop a multiphasic approach to alleviate the occurrence of ARD in apple orchards under South African conditions (Tewoldemedhin *et al.,* 2011).

A consequence of commercial fumigation of replanted soils with methyl bromide is that both advantageous and pathogenic soil biota is eradicated, rendering the soil a "lifeless" growing medium (Cabrera *et al.,* 2015). Fumigation is then typically followed by an inorganic/chemical fertilizer application, in addition to irrigation and pest and disease management to promote optimal plant performance (Pecina *et al.,* 2016). When implementing this approach, MB fumigated soils still resulted in higher plant performance compared to when orchards are established on untreated ARD soils.

Biostimulants is defined by the European Biostimulants Industry Council (EBIC), as products containing microorganisms and/or substances that stimulate natural processes to enhance nutrient uptake and efficiency within the plant, whilst increasing tolerance to abiotic stress, and improving crop quality when applied to the rhizosphere (Brown and Saa, 2015; Du Jardin, 2015; Rouphael *et al.,* 2020). Biostimulants may include several type of components, ranging from seaweed extracts to microbial inoculants, humic and fulvic acids, protein hydrolysates and amino acids (Calvo *et al.,* 2014; Rouphael *et al.,* 2020). These products provide a distinct benefit over biological control, in that an induced resistance against plant diseases is achieved through improved plant growth, stress tolerance and improve nutrient absorption by the plant roots *(*Calvo *et al.*, 2014; Rouphael *et al.*, 2020).

A variety of commercial biostimulant products have been tested under South African conditions (Aremu *et al.*, 2015; Lötze and Hoffman, 2016; Shereni, 2019; Stirk *et al.*, 2014) on different vegetable and horticultural crops including slim amaranth, garden tomatoes, winter squash (Ngoma *et al.,* 2013), soybean (Rathore *et al.,* 2009), pineapple lily (Aremu *et al.,* 2016), apricots (Fathy *et al.,* 2010), olives (Bartolini *et al.,* 1993; Sharma *et al.,* 2011), apples (Sahain *et al.,* 2007) and citrus, plums and pears (Shereni, 2019). Yet, the use of biostimulants to mitigate transplant shock in apples after fumigation under South African conditions remains unexplored.

The aim of this study was to determine whether biostimulants, applied once to newly established apple trees in a fumigated soil, could reduce transplant shock and improve the performance of vegetative aerial parameters of apple trees under South African conditions.

Plant performance was quantified by recording various physiological stress indicators as well as determining aerial vegetative growth in 'Granny Smith'/MM109, trees during two consecutive seasons. This investigation forms part of a more comprehensive study that also considered treatment effects on the root growth dynamics and soil microbial activity, to develop a more holistic view on the role biostimulants may play to restore soil health following fumigation to control ARD under South African conditions.

2. MATERIALS AND METHODS

Site Description

The trial was conducted on newly planted *Malus domestica* 'Granny Smith' trees on MM109 rootstock, in a commercial orchard on Lovenstein farm (34° 4' 55'' S; 10° 4' 12'' E) in Vyeboom, Western Cape, South Africa. Trees were planted in October 2016, with planting distance 5 m × 2 m, with a north to south facing row direction. The orchard was fumigated by a commercial company, BioScience Research in September 2016, approximately two weeks prior to planting, using a combination of chloropicrin and 1, 3-D-Chloropicrin at the recommended dosage and standard protocols (BioScience Research, 6 Pastorale Street, Durbanville).

Treatments

Treatments were applied to the roots of the trees at planting, according to the protocol of the commercial products, by following one of three methods: i) dipping the roots in treatment solution before planting, followed by drenching the rhizosphere with water directly after planting, ii) applying the treatment as a soil drench near the roots of the tree, directly after being planted or iii), the applying the treatment in the planting hole, before planting (compost) (Fig. 1). Seven treatments (including the control, which consisted of a fumigation treatment only) were applied and replicated 10 times, in a randomised complete block design, with single trees serving as experimental units. A full description of the method of application for each treatment is summarized in Table 1 (Paper 1). At planting, trees were watered with approximately 15 - 25 litres of water per tree by hand twice a week as the micro-irrigation was only installed approximately one month after planting. From January 2018, the ridge was covered by a thin mulch (Fig. 3). Herbicides and pesticides were applied throughout the two seasons following standard orchard practices.

Soil amendments and management practices

Soil preparation and management were performed according to standard recommendations based on a chemical soil analysis. Thereafter, the soil was ridged to approx. 50 cm x 200 cm in the planting row. A combination of organic and anorganic products were applied as follows: 'Enhancer' (InteliCHem Pty Ltd.,) at 1.2 kg. tree^{-1} was added to the soil at planting, whilst Maxifos (Omnia Nutriology®) and KCl was applied at 500 and 300 kg.ha-1 respectively, in May 2016. Controlled release fertilizer was applied at 200 kg.ha^{-1} in December 2016 and again in November 2017. From November 2016 to February 2017, 240L compost-tea (Ecosoil (PTY) LTD., Longshadows Farm, Viljoenshoop Rd, Elgin, Western Cape) was applied in monthly intervals.

Data collection

Vegetative parameters

Leaf Nutrient Analysis. Five healthy leaf samples per treatment were collected according to standard procedures after the shoot flush had been completed, on 25 February 2017 and 16 February 2018, and sent to a commercial laboratory Bemlab (Pty Ltd, Strand) for standard mineral analyses.

Stem diameter. Stem diameter was recorded on the day of planting and thereafter annually, with an electronic Vernier calliper, approximately 10 cm above the rootstock union on the 22nd of October 2016 and the 30th of October 2017.

Shoot growth. Shoot length was recorded annually, after completion of summer flush, using a measuring tape on two representative one-year-old shoots in the tree canopy for all treatments and both seasons (Fig. 2) measured on the 25th of February 2017 and the 12th of February 2018.

Total soluble sugars (TSS). The TSS of the leaves from 35 trees (5 replicates per treatment) were determined by compressing two healthy leaves per tree in leaf-crushing canister (Nulandis, Paarl) on 19 April 2017 and again on 11 April 2018 for comparison to the norm threshold value for apple trees (van Zyl, 2016). Leaf TSS was measured as % Brix by applying a drop of the composite juice on a hand-held refractometer (ATAGO, PR 32, USA).

Physiological Parameters

Photosynthetic capacity and stomatal conductance. Photosynthesis and stomatal conductance were recorded on two healthy, fully exposed mature leaves per tree, with five replicates per treatment using an open gas exchange system infrared gas analyser (IRGA) (Li-6400 portable photosynthesis system, Li-Cor Inc., Lincoln, NE, USA). The flow rate and light intensity were set to 500 µmol.s^{-1} and 1500 µmol.m^{-1}.s^{-1} respectively, whilst the ambient air temperature ranged from 26 °C to 33 °C.

Stem Water Potential (SWP). Midday stem water potential (MPa) was measured with a Scholander pressure chamber (PMS Instruments, USA) on 14 March 2017 and again on 29 March 2018 to determine water potential as indication of water stress. Two healthy, fully expanded, mature, sun-exposed leaves per tree were selected for this purpose, with five replicates per treatment. The leaves were enclosed in a plastic film bag covered with aluminium foil an hour before recording. After an hour, each leaf was cut off at the petiole and placed in the pressure chamber, with the cut end protruding through the seal, to determine stem water potential. Pressure was applied to the chamber using nitrogen gas until a droplet of the leaf sap became visible under a magnifying glass (Scholander pressure chamber, PMS Instruments, USA).

Statistical Analysis

Statistical analyses were performed using a two-way analysis of variance (ANOVA), generalized linear model (GLM) procedure in SAS 9.4 (SAS Institute Inc. 2004, Cary, USA). Means were separated by Least Significant Differences (LSD) when significant differences occurred at a 5 % confidence level ($p \leq 0.05$).

3. RESULTS

Vegetative parameters

Leaf nutrient analysis. Nutrient concentrations in the leaves differed between the two seasons, however for both seasons the leaf nutrient analysis did not show any significant differences between treatments for either the macro- or micronutrients (Tables 1; 2). No visual symptoms of deficiency or toxicity were observed during either season (personal observation, M. Webber). However, nitrogen (N), phosphorus (P), sodium (Na) and manganese (Mn) levels in

2017 exceeded the commercially recommended threshold values, whereas the levels for potassium (K), copper (Cu), boron (B) and zink (Zn) concentrations were closer to the minimum commercial levels. During Jan 2018, concentrations for N, P and Mn still exceeded the maximum threshold, whilst that for K, Mg, Na, Fe and Cu were verging on the higher end of the range. Ca levels, however, were noticeably at the lower end of the range for both seasons, as was also the case for Zn. B levels were at the lower end of the range, specifically for the 2018 season, but did not differ significantly from the other treatments. A general trend of higher nutrient values for the L-AA treatment in 2017 than in 2018 was observed (Tables 1; 2).

Stem Diameter. No significant differences in stem diameter were recorded between the various biostimulant treatments, during either of the two seasons (Table 3). Stem diameters increased with an average of 9 mm between seasons, which provides evidence for active tree growth during the observation period (Table 3).

One-year shoot growth. No significant differences were found between any of the biostimulant treatments over the course of two growing seasons (Table 3). Average shoot growth increased from 33.1 mm (2017) to 42.4 mm (2018), again indicating positive tree growth.

Leaf total soluble sugars (TSS). No significant differences between treatments were reported for leaf TSS within the season average leaf TSS of 3.3 % in the 2016/17 season was well below the threshold or stress value of 6 % as reported by (Van Zyl, 2016). Leaf TSS in the 2017/18 season however showed higher average values of 7.7 % (Table 4).

Physiological parameters

Photosynthetic capacity and stomatal conductance. No significant differences in photosynthetic capacity expressed as A_{max} (Table 4) or stomatal conductance (Table 5) was noted between treatments, irrespective of season.

Stem water potential (SWP). There were no significant differences between treatments for stem water potential in either season, at the 5% confidence level (Table 5). However, in the 2017/18 season, a trend was noted where the Trich 1 treatment showed the most negative stem water potential, and therefore the most water stress, compared to all the other treatments which varied with a range of -7.45 to -8.39 MPa (Table 5).

4. DISCUSSION

Plant nutrient uptake is influenced by drought, soil properties, root morphology and cultural management practices (Heydari and Misaghi, 2011). Previous studies noted the role of biostimulants in promoting nutrient uptake in various crop types such as apple (Sahain *et al.*, 2007), citrus (Shereni, 2019) and maize (Alam, 2013). In particular, the application of plant growth promoting rhizobacteria (PGPR) to 'Granny Smith' trees was noted to coincide with an increase N, P, K, Ca, Fe, Mn, and Zn (van Schoor *et al.*, 2008). In our study, no significant differences between treatments were observed over two consecutive seasons. However, a trend occurred where most of the foliar nutrient levels were on the higher end of the recommended range or exceeded the concentration from establishment. This is indicative of an unrestricted uptake of mineral nutrients, except for Ca, which was found to be deficient in 2018.

The excessive concentrations of N and P that occurred in both seasons and across treatments, may be partly contributed to the mode-of-action of plant growth promoting bacteria (PGPB) which includes N_2-fixing and P-solubilisation (Alam, 2013). PGPB was present in all the treatments in our study, except for the untreated Control. However, as the N and P levels in the Control did not differ significantly from levels measured in leaves of the treatment trees, a more plausible explanation for the excessive N and P foliar levels can be the contribution and uptake associated with the commercial fertilizer application.

The deficiency of Ca in the second season was unexpected. One possible explanation is that insufficient Ca was applied during amelioration, yet this was not confirmed with a soil analysis. Another possibility for the low Ca levels is a lack of white root tips and insufficient soil moisture, which is required for optimum Ca uptake. However, sufficient root growth was confirmed during both seasons, which should have ensured sufficient Ca uptake (Paper 1). Yet, water stress was experienced during the second season and this may have contributed towards limited uptake of Ca from the soil, as a result of reduced transpiration, mass flow and therefore low translocation of Ca in the plant (Danner *et al.*, 2015).

Stem water potential is an accepted estimator of plant water status (Choné *et al.*, 2001). In our study, no significant differences were found between treatments during either of the seasons. Of interest, however, is that the SWP for the second season exceeded the stress reference value provided for young apple trees of 1.04 and -1.46 MPa (De Swaef *et al.*, 2009). This is concurrent with the limited rainfall recorded during the 2017/18 seasons, as well as reported results following implementation of deficit irrigation as is typically applied during drought conditions (Spann and Little, 2010). The SWP status thus provides evidence of the conditions of the water stress that was experienced during the second season, as was also confirmed by lower soil moisture levels that were recorded (Paper 1).

Stomatal conductance and photosynthetic capacity provides information on the rate at which leaves are able to fix carbon during photosynthesis (Kattge *et al.*, 2009). Photosynthetic rate is influenced by solar irradiance, leaf temperature, vapour pressure deficit and plant water status (Massonnet *et al.*, 2007). In our study, no significant differences were observed between treatments over the two growing seasons. However, a trend indicated the lowest photosynthetic capacity and stomatal conductance in the Plant Extract treatmen, as well as highest SWP, during both seasons, indicating conditions of water stress which may have limited optimal photosynthesis for this treatment, especially during the second season (De Swaef *et al.*, 2009; Fanwoua *et al.*, 2014; Galindo *et al.*, 2018; Šircelj *et al.*, 2007).

Stem diameter and shoot length measurements can be used to quantify aerial vegetative performance, where a larger trunk diameter can be considered indicative of stronger tree growth (Miller, 1983). In our study, the general increase in vegetative parameters confirmed growth from the first to second season. However, no significant differences in stem diameter between treatments, in either of the seasons were detected. A similar lack of significant effects of treatments on aerial growth in apple trees were also reported by Yao *et al*. (2006), who found that pre-plant treatments after fumigation had no effect on tree yield and growth. However, the effect of competition for assimilates by tree roots is another possible avenue to explore before final conclusions are drawn on above-ground tree performance.

Roots are the primary sinks of assimilates in young non-bearing trees (Cheng *et al.*, 2008). Therefore, a lower concentration of assimilates would be expected in the leaves of such trees at the beginning of the season. An easy, fast, and practical method to quantify assimilate accumulation in leaves is to determine the total soluble solids of those specific leaves by means of a refractometer (Harrill, 1994). TSS for apples using this method are divided into four classes: poor (6 %), average (10 %), good (14 %) and excellent (18 %) (Harrill, 1994), where the lower ‘poor’ class served as an indication of stress. In our study, leaf TSS values ranged

from 2.29 to 4.31% Brix for the 2017 season, although no significant differences in leaf TSS were found between treatments. In the 2018 season, higher TSS within a range of 6.72 to 8.68 % Brix were recorded between treatments, again with no significantly between treatments. However, this elevated range noted in the second season compared to the first season could be indicative of drought stress (Galindo *et al.*, 2018) as was also suggested by the SWP data.

The impact of biostimulants on perennial plants is usually reported over a longer period, from two years onwards, compared to abiotic/chemical treatments that may yield shortterm results. Therefore, the expectation to differentiate between treatments within two seasons may have been premature. Yet, it is highly unlikely that a biostimulant treatment could have an extended effect on stress conditions more than 12 months after a single soil application, unless a significant effect occurred during the first season. In our study, no indications of a significant effect of any of the treatments on the vegetative aerial parameters of tree performance was noted during the first season which could have primed the trees to show significant differences in growth in the next season.

Stunted growth, induced by fumigants including chloropicrin were reported in cotton, corn, citrus and avocado seedlings (Menge, 1982; Plenchette *et al.*, 1983). Such stunting is related to a combination of factors that limit and delay the root growth development, which then result in nutrient deficiency (Šircelj *et al.*, 2007), as opposed to the absence of beneficial soil microorganisms or the presence of pathogenic soil borne organisms (Linderman *et al.*, 2007). In our study, root growth did not appear to be visually stunted. This observation was supported by adequate mineral nutrient levels, as well as vegetative indicators, that showed positive increases in stem diameter and shoot growth in both seasons.

An overall increasing trend in total root number across all treatments, throughout the first season (Paper 1), supported findings of Van Zyl (2016) and Lötze *et al*. (2018) where continuous root growth in young, non-bearing apple trees were noted throughout the year. Such continuous root growth may compete as stronger sinks for the allocation of assimilates in young trees as opposed to the shoot development (Irving, 2015). Such asynchronized patterns in growth between roots and above ground canopy were also observed by Ma *et al*. (2013) in 'Red Fuji' apple trees. In our study, significant differences between treatments for the various root parameters on specific evaluation dates and soil depths (Paper 1), however did not result in any significant differences in vegetative growth between treatments, over two seasons. It can thus be assumed that the differences between treatments, which resulted in differences in various root growth parameters did not lead to a differential root to shoot competition expression,

which would then have resulted in significant differences in vegetative growth, over the two seasons.

5. CONCLUSION

Biostimulants enhance tree performance under abiotic stress conditions like drought and/or biotic stresses such as pest and disease pressure. The objective of this study was therefore to determine the effect of biostimulant application at establishment following fumigation, on the aerial, vegetative performance of the young apple trees. In our study, the focus was on the mitigation of transplant shock and the microbial population recovery after fumigation, as an induced stress condition. However, an unforeseen drought was also experienced during the study, which created an additional stress condition to that of fumigation after establishment.

Results showed no significant differences in vegetative growth or physiological parameters were recorded between treatments, for either season. Thus, despite that the effect of the drought was confirmed during the second season by means of physiological parameters, none of the biostimulant treatments were able to reduce the stress preferentially nor could they increase the vegetative growth significantly compared to the Control, year after application. This finding was unexpected, as root growth parameters indicated significant differences between treatments within the first season, with a clear differentiation between the biostimulants, based on the mode-of-action of treatments. In addition, drought stress presented an ideal opportunity for the Mycorrhiza treatment to deliver the benefit of increased water uptake and the resulting lower plant stress, promoting growth, as reported by Asrar and Elhindi (2011). The Mycorrhizae treatment did result in a significantly higher root volume than the other treatments (Paper 1). Yet, the expected transferred benefit for enhanced shoot growth was not observed. Nevertheless, no treatment had a negative effect on tree growth.

In this study, where environmental conditions following application of treatments were harsh and unfavourable for the establishment of microbial populations, the application of most treatments (Compost, Trich 1, Trich 2, L-AA and Mycorrhizae) only occurred once off at establishment, and in the case of Plant Extract, monthly applications continued for a six-month period. It is therefore proposed that in a future, the application of the promising biostimulant treatments be continued for an extended period after establishment, to enhance the initial positive reaction on root growth and continue to promote the recovery of the soil microbial environment for support transplant recovery and the translation thereof into improved tree performance.

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7. TABLES AND FIGURES

Table 1: Treatment details for the 'Granny Smith'/MM109 apple trial on Lovenstein farm, Vyeboom, for biostimulant applications during establishment in October 2016, after soil fumigation

Treatment	Product/ Supplier	Application method
Control (water) (Fumigation only)	Control	-Add water to the tree at planting
Compost Cow manure and garden refuge	Stellenbosch University	-Apply in the planting hole at planting
Mycorrhiza	Mycogel from Kimitec/ Wenkem SA (www.wenkem.co.za)	-0.5 ml of product per tree, applied at planting or within 3-4 days of planting -No phosphates or pesticides should be applied within 14 days after application, only use of organic fertilizer
Plant Extract Plant extracts, PGPB - Bacillus Bacteria (95%), microbes & naturally occurring organic-soluble humates	Aqua Clean SA Sludge Abate Blue Planet SA (www.blueplanet-sa.com)	-150 ml of well-mixed product added near tree roots -Applied with enough water to spread the product
Trichoderma 1 (Trich 1) Contain 8 different isolates of Trichoderma: -*T.asperellum* (3) -*T. hamatum* (1) -*T. atroviride* (2) -*T.harzianum* (2)	Bio-Tricho Liquid Agro-Organics (www.agro-organics.co.za)	-Submerge tree roots should in a solution of spore suspension of 400 ml.100L^{-1} product in water -Soak for 10 minutes or longer before transplanting -Commercial soil application at 1L. ha^{-1}
L-Amino Acids (LAA) Contains 19 synthesized L-amino acids, oligo-peptides and nutrients	Aminostim from Bioscience Research (www.brsa.com)	-A liquid formulation mixed with water & applied by means of foliar spray at 0.5L– 1L. ha-1), -Drip irrigation at 1.5L to 3L. ha-1 or -Apply in hydroponics at lower rates to young plants and during flowering stages -Applied commercially as a liquid through irrigation every 4 weeks at 25ml per tree or 15L.ha-1
Trichoderma 2 (Trich 2) Contains 4 selected species of *Trichoderma* endophytes	Excalibur Gold ABM Africa (www.abm1st.com)	-Submerged trees into a solution of the product or as a drench (500 g.ha^{-1})

Table 2: Mineral analysis (n=5) for 'Granny Smith' apple leaves collected on Lovenstein, Vyeboom, February 2017, following 3 months after biostimulant treatments were applied to restore soil health following fumigation.

Treatment	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Na (ppm)	Mn (ppm)	Fe (ppm)	Cu (ppm)	Zn (ppm)	B (ppm)
Control	2.9 ns	0.2 ns	1.1 ns	1.1 ns	0.3 ns	1238.4 ns	127.2 ns	174.4 ns	6.0 ns	35.4 ns	27.6 ns
Compost	3.0	0.3	1.3	1.2	0.3	942.6	100.6	133.4	7.4	31.8	31.2
Mycorrhiza	3.1	0.3	1.7	1.4	0.4	824.2	101.6	184.4	9.0	37.4	37.8
Plant extract	2.8	0.3	1.3	1.3	0.3	747.6	120.0	193.6	7.0	25.8	24.8
Trichoderma 1 (Trich 1)	3.2	0.3	1.5	1.4	0.3	812.4	130.8	236.8	6.6	33.8	35.6
Trichoderma 2 (Trich 2)	2.9	0.3	1.5	1.4	0.3	866.6	110.0	184.8	8.4	32.0	36.4
L-Amino Acids (L-AA)	3.2	0.3	1.6	1.4	0.4	1206.2	128.0	202.8	7.6	31.0	37.4
Industry norm	*2.05 - 2.54*	*0.14 - 0.18*	*1.15 - 1.53*	*1.13 - 1.77*	*0.30 - 0.40*	*500**	*20 - 94*	*94 - 177*	*4-9*	*30 - 50*	*24 - 39*
P	**0.6780**	**0.5209**	**0.3955**	**0.9228**	**0.9343**	**0.5928**	**0.8737**	**0.1934**	**0.5008**	**0.3966**	**0.3478**

*The industry norms according to Bemlab Pty Ltd.

Table 3: Mineral analysis of 'Granny Smith' on Lovenstein, Vyeboom (n=5), in February 2018, following 15 months after initial biostimulant treatments were applied after fumigation, analysed by the commercial laboratory Bemlab Pty Ltd.

Treatment	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Na(ppm)	Mn (ppm)	Fe (ppm)	Cu (ppm)	Zn (ppm)	B (ppm)
Control	2.7 ns	0.28 ns	1.0 ns	1.0 ns	0.4 ns	574.6 ns	107.0 ns	163.4 ns	9.4 ns	32.2 ns	27.8 ns
Compost	2.8	0.29	1.0	1.2	0.4	453.2	139.0	154.8	8.2	29.2	26.2
Mycorrhiza	2.9	0.25	1.0	1.0	0.4	488.8	91.8	150.8	9.0	30.8	27.2
Plant extract	2.8	0.23	2.0	0.9	0.4	544.4	108.0	161.8	8.2	24.8	27.4
Trichoderma 1	2.6	0.30	1.0	1.0	0.4	451.0	99.8	201.6	8.6	29.8	27.4
Trichoderma 2	2.7	0.27	1.0	1.1	0.4	533.4	166.0	154.6	7.8	26.2	27.0
L-Amino Acids	3.0	0.27	1.0	1.0	0.4	696.4	130.0	143.0	8.8	30.8	28.0
Industry norm	*2.05 - 2.54*	*0.14 - 0.18*	*1.15 - 1.53*	*1.13 - 1.77*	*0.30 - 0.40*	*500**	*20 - 94*	*94 - 177*	*04-09*	*30 - 50*	*24 - 39*
P	**0.2361**	**0.8818**	**0.7377**	**0.5917**	**0.7017**	**0.4879**	**0.4224**	**0.1256**	**0.0900**	**0.3159**	**0.9598**

* The industry norms according to Bemlab Pty Ltd.

Table 3: Stem diameter (mm) and shoot growth (cm) for 'Granny Smith' apple trees on Lovenstein, Vyeboom for two consecutive seasons after biostimulant treatments were applied to restore soil health following fumigation.

	Stem diameter (mm)			Shoot growth (cm)	
Treatment	**2017**	**2018**	**Δ 2017/2018**	**2017**	**2018**
Control	18.2 ns	28.0 ns	9.8	37.9 nss	38.2 ns
Compost	18.0	27.3	9.3	36.2	47.2
Mycorrhiza	17.9	27.1	9.2	29.8	45.7
Plant extract	18.3	27.0	8.7	40.2	47.5
Trichoderma 1	17.2	25.8	8.6	33.9	39.3
Trichoderma 2	17.9	26.5	8.6	26.9	36.9
L-Amino Acids	19.0	28.2	9.2	26.5	41.8
P	**0.6500**	**0.5827**		**0.8202**	**0.6466**

Table 4: Percentage leaf total soluble solids (TSS) and photosynthetic capacity (A_{max}) measured in 'Granny Smith'/MM109 apple tree leaves from Lovenstein, Vyeboom during the 2016/17 and 2017/18 seasons, following the application of a range of selected biostimulant treatments to restore soil health after fumigation.

	TSS (% Brix)		A_{max} (µmol.m^2.s^{-1})	
Treatment	**2017**	**2018**	**2017**	**2018**
Control	3.88 ns	8.24 ns	18.62 ns	15.86 ns
Compost	3.43	7.56	18.64	14.92
Mycorrhiza	4.31	7.58	19.08	16.08
Plant extract	2.29	8.58	17.48	13.32
Trichoderma 1	2.67	6.82	19.57	17.58
Trichoderma 2	3.44	8.68	20.70	15.07
L-Amino Acids	2.57	6.72	18.28	16.37
P	**0.1669**	**0.5008**	**0.6780**	**0.2509**

Table 6: Stomatal conductance and stem water potential (SWP) of young 'Granny Smith'/MM109 apple trees from Lovenstein, Vyeboom during the 2016/17 and 2017/18 seasons, following the application of biostimulant treatments.

	Stomatal conductance ($mmol.m^2.s^{-1}$)		Stem water potential (MPa)	
Treatment	**2017**	**2018**	**2017**	**2018**
Control	0.15^{ns}	0.28 ^{ns}	-1.14 ^{ns}	-8.14^{ns}
Compost	0.16	0.27	-1.31	-8.04
Mycorrhiza	0.15	0.27	-1.19	-7.45
Plant extract	0.13	0.21	-1.25	-7.67
Trichoderma 1	0.16	0.34	-1.27	-14.25
Trichoderma 2	0.17	0.27	-1.34	-7.84
L-Amino Acids (L-AA)	0.15	0.28	-1.19	-8.39
P	**0.555**	**0.329**	**0.656**	**0.062**

Table 7: Variation (n=4) in soil composition and texture taken from ridges at two depths on Lovenstein, Vyeboom, immediately prior to establishment of 'Granny Smith'/MM109 in October 2016. Blocks represent different soil profiles throughout the orchard that coincide with the trial layout and indicate the big soil texture variation in the layout.

Site	**Soil Depth (cm)**	**Soil organic carbon (%)**	**Stone (%)**	**Clay (%)**	**Silt (%)**	**Sand (%)**	**$pH_{(KCl)}$ (1)**	**P (Bray, ppm)(2)**
Block A	0-15	1.44	18	13	7	80	5.5	71
	15-30	1.17	17	13	7	80		
Block B	0-15	1.35	19	9	4	87	5.5	44
	15-30	1.18	15	16	4	80		
Block C	0-15	1.21	14	16	7	77		102
	15-30	0.92	15	9	7	84	5.5	
Block D	0-15	0.61	46	20	11	69		*(3)
	15-30	0.47	45	20	15	65	5.5	

[1]Average pH(KCl) based on composite sample.
[2]Phosphorus present should measure >30 ppm as per recommendation.
[3]Represents missing values.

Figure 1: The treatment application methods that was conducted on young ‘Granny Smith’/MM109 trees at planting (16 October 2016) on Lovenstein, Vyeboom, Western Cape included: (a) submerging the tree roots in the treatment solution immediately prior to planting for approximately 10 minutes; (b) applying the treatment to the rhizosphere of the tree at planting; and (c) applying the treatment directly to the tree roots at planting.

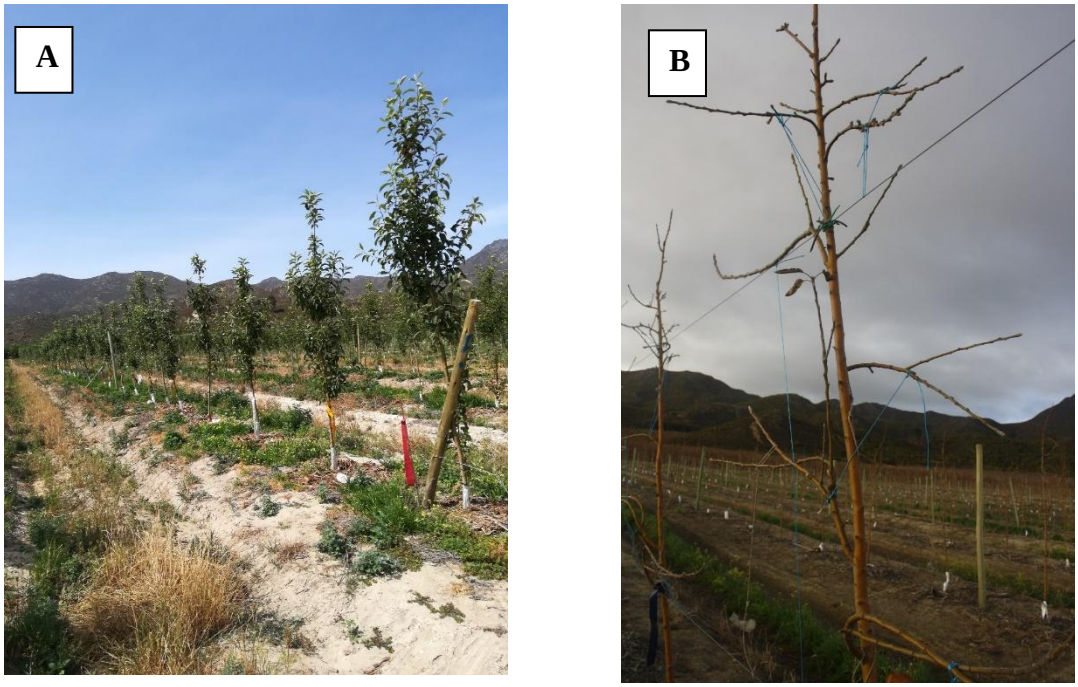

Figure 2: Vegetative development of young ‘Granny Smith’/MM109 apple trees planted in fumigated soil during October 2016 represented 15 months after planting (a) (January 2018) and 23 months after planting (September 2018) on Lovenstein farm, Vyeboom, Western Cape.

Figure 3: Minimal mulching applied to Young ‘Granny Smith’/MM109 apple trees planted in fumigated soil during 15 months after planting (January 2018) on Lovenstein farm, Vyeboom, Western Cape.

GENERAL DISCUSSION AND CONCLUSION

The aim of this study was to determine the effect of biostimulants on tree performance after establishment in a fumigated ARD infected orchard and consequently provide a better understanding of the plant-soil-biostimulant interaction and site-specific reactions.

In paper 1, the effect of biostimulants on root growth dynamics showed significant differences at establishment after limited applications of biostimulants. A trend indicated that the effect of the different treatments varied according to the mode of action of the biostimulants. It was evident from this study that most significant differences in root dynamics were observed in the topsoil layers of the profile. A definite physiological effect on root growth and architecture was confirmed with Mycorrhizae mainly affecting root volume and *Trichoderma* spp. (Trich 1 and Trich 2) affecting root length and number, under conditions of limited application of treatments. Evaluation of seasonal trends for root dynamics during 2016/17 showed three distinct reaction patterns for treatments with clustering results as follows: Mycorrhizae, *Trichoderma* formulations (Trich 1 and Trich 2) and Others (L-AA, Plant Extract and Compost). No treatment, however, consistently outperformed one another and results varied between treatments over time. This confirmed the influence of site-specific factors such as unfavourable soil texture and limited soil moisture on consistent results of the efficacy of different biostimulant treatments. Applying biostimulants at the correct dosage and optimal timing is critical for optimal efficacy and was a limitation during this study.

Significant differences between treatments in root dynamics (Paper 1) were not sufficient to quantify successful soil microbial rehabilitation after fumigation. Two distinct modes of action were evident when the treatments considered stimulating microbial recovery (L-AA and Plant Extract) or promoting root growth dynamics (Mycorrhizae). Last mentioned was also confirmed by the results of paper 1. Findings also confirmed that not all microorganisms were eradicated following fumigation, when the presence of *Trichoderma* spp. was found on the roots of the untreated Control, as well as the treatments formulated without *Trichoderma* spp. in Paper 2. *Trichoderma* formulated treatments did show the highest occurrence of *Trichoderma* spp., as well as an increased total root number throughout the season. This is concurrent with the results seen in Paper 1 and confirms once more the effect of biostimulants on root growth dynamics.

Paper 3 showed no significant differences between treatments regarding vegetative growth over the two seasons. An adjustment of the application protocol should be evaluated in future studies.

The overall findings of this study confirm the effect of biostimulants on root growth dynamics and rehabilitation of soil microbial communities after fumigation under field conditions. Our results justified future research with adapted methodology to obtain consistent results in field trials to ultimately apply the correct biostimulant in the correct concentration and frequency to obtain sustainable growth, rehabilitation, and production in the long term.

ANNEXURES

Annexure A: Average root length (ARL) (mm) for 'Granny Smith'/M109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap! software for the biostimulant treatments at four soil depths (levels) during season 1 (2016/17). Level 1 (45 – 60 cm) below the surface, 2 (30 – 45 cm), 3 (15-30 cm) and 4 (0 – 15 cm). * Represents data not available.

Average Root Length (mm), Season 1 (201617)										
Level	**Treatment**	**15 Dec 2016**	**01 Jan2017**	**15 Feb 2017**	**14 Mar 2017**	**19 Apr 2017**	**18 May 2017**	**12 Jul 2017**	**30 Aug 2017**	**02 Oct 2017**
1 (45-60 cm)	Control	18.334 ns	27.201 d	26.420 ns	33.103 ns	33.026 ns	31.330 ns	35.880 ns	35.000 ns	33.870 ns
	Compost	47.684	50.219 ab	50.330	51.090	50.374	50.580	50.390	49.850	49.850
	Mycorrhiza	27.263	52.429 a	59.210	53.487	56.568	48.712	53.850	54.800	66.970
	Plant Extract	45.019	47.111 ab	46.740	43.652	43.981	43.908	43.950	45.400	45.370
	Trichoderma & Amino Acids	36.902	35.197 cd	39.480	44.661	44.629	44.885	44.030	49.210	49.560
	Trichoderma 1	40.653	40.394 bc	41.550	42.209	41.721	41.176	37.200	38.340	*
	Trichoderma 2	39.365	43.522 abc	44.160	45.379	45.918	45.660	38.750	40.620	40.920
	P-Value	**0.0752**	**0.0036**	**0.2033**	**0.4796**	**0.1728**	**0.2348**	**0.6264**	**0.7856**	**0.3554**
2 (30-45 cm)	Control	25.520 ns	31.747 ns	32.139 ns	36.642 ns	37.500 ns	37.153 ns	31.770 ns	32.360 ns	32.806 ns
	Compost	45.480	50.712	50.312	52.145	52.370	52.344	48.330	49.390	49.678
	Mycorrhiza	31.420	56.142	54.849	47.110	50.340	52.702	64.770	52.640	52.644
	Plant Extract	43.280	44.244	47.503	42.762	42.850	42.215	43.530	44.450	44.521
	Trichoderma & Amino Acids	53.880	48.477	49.018	51.419	51.560	51.641	54.330	52.700	52.564
	Trichoderma 1	41.080	43.709	43.793	43.015	43.460	44.776	46.860	31.500	*
	Trichoderma 2	42.830	44.031	44.369	46.842	42.690	43.227	42.980	42.720	42.819
	P-Value	**0.4478**	**0.1548**	**0.3407**	**0.6881**	**0.7529**	**0.5287**	**0.5502**	**0.3115**	**0.3000**
3 (15-30 cm)	Control	38.830 ns	37.401 ns	37.802 ns	39.860 ns	40.650 ns	38.410 ns	42.033 ns	41.608 ns	42.072 ns
	Compost	52.620	54.019	52.724	55.880	56.640	55.930	42.423	47.129	49.299
	Mycorrhiza	37.970	61.565	58.022	50.680	48.700	62.740	55.213	60.621	52.011

	Plant Extract	48.540	49.941	51.642	53.020	52.570	51.660	51.875	52.244	52.541	
	Trichoderma & Amino Acids	54.590	56.384	57.166	57.040	55.720	57.340	53.353	56.287	57.873	
	Trichoderma 1	36.950	39.930	40.853	40.730	41.250	40.080	37.750	39.448	*	
	Trichoderma 2	41.630	49.296	49.615	50.940	46.360	46.510	39.062	45.048	46.449	
	P-Value	**0.7006**	**0.0854**	**0.2210**	**0.9035**	**0.9348**	**0.4139**	**0.3679**	**0.2223**	**0.6501**	
4 (0-15 cm)	Control	28.680 b	32.070 ns	33.940 ns	35.990 ns	38.134 ns	36.780 ns	40.900 ns	41.030 ns	41.020 ns	
	Compost	35.740 b	37.470	38.120	42.240	42.340	42.310	38.790	44.030	43.900	
	Mycorrhiza	73.340 a	60.760	59.500	64.240	43.468	45.650	54.090	51.900	51.570	
	Plant Extract	38.260 b	41.180	26.410	39.510	42.929	42.000	35.160	38.350	32.940	
	Trichoderma & Amino Acids	30.780 b	46.150	44.880	42.670	36.991	40.190	42.110	40.350	40.250	
	Trichoderma 1	38.350 b	39.750	42.010	43.050	42.965	42.530	33.600	36.360	*	
	Trichoderma 2	39.770 b	42.890	45.230	43.140	40.622	40.380	40.190	41.070	41.440	
	P-Value	**0.0264**	**0.3109**	**0.5227**	**0.5559**	**0.9822**	**0.9916**	**0.8435**	**0.8466**	**0.8021**	

[ns] Alphabetic letters show significant differences in values when significant differences occurred at a 5% confidence level (P<0.005)

Annexure B: Average root area (ARA) (mm^2) for 'Granny Smith'/M109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap! software for the biostimulant treatments at four soil depths (levels) during season 1 (2016/17). Level 1 (45 – 60 cm) below the surface, 2 (30 – 45 cm), 3 (15-30 cm) and 4 (0 – 15 cm). * Represents data not available.

Average Root Area (mm^2), Season 1 (201617)																			
Level	**Treatment**	**15 Dec 2016**		**01 Jan 2017**		**15 Feb 2017**		**14 Mar 2017**		**19 Apr 2017**		**18 May 2017**		**12 Jul 2017**		**30 Aug 2017**		**02 Oct 2017**	
1 (45-60 cm)	Control	38.6	ns	50.0	b	48.7	b	63.1	b	61.3	b	57.7	s	62.4	b	60.1	b	58.3	b
	Compost	97.6		95.9	b	95.8	b	100.5	b	98.95b	b	99.6		89.8	b	94.5	b	94.2	b
	Mycorrhiza	181.8		1238.3	a	616.7	a	610.4	a	601.9	a	449.4		788.1	a	844.0	a	1811.1	a
	Plant Extract	91.3		99.5	b	95.6	b	76.9	b	76.4	b	75.9		72.0	b	74.4	b	74.4	b
	Trichoderma & Amino Acids	76.4		61.3	b	71.5	b	75.6	b	74.5	b	74.8		75.5	b	84.8	b	85.5	b
	Trichoderma 1	66.7		67.3	b	73.3	b	76.8	b	76.3	b	75.4		64.0	b	66.5	b	*	
	Trichoderma 2	67.0		78.1	b	80.9	b	82.8	b	83.6	b	83.4		62.8	b	67.5	b	68.1	b
	P-Value	**0.6929**		**<.0001**		**<.0001**		**<.0001**		**<.0001**		**0.1239**		**<.0001**		**0.0015**		**0.0254**	
2 (30-45 cm)	Control	47.2	ns	59.0	b	57.6	b	61.7	s	62.6	b	63.0	b	42.4	b	46.2 s	s	47.7	b
	Compost	88.0		101.0	b	99.9	b	98.8		99.4	b	99.2	b	100.6	b	106.3		106.4	b
	Mycorrhiza	637.2		852.3	a	760.4	a	766.3		893.8	a	790.5	a	866.2	a	645.2		1010.6	a
	Plant Extract	98.4		80.0	b	87.6	b	71.9		71.5	b	69.3	b	74.6	b	75.3		76.1	b
	Trichoderma & Amino Acids	104.1		83.8	b	84.1	b	91.5		90.8	b	89.8	b	99.6	b	97.4		96.6	b
	Trichoderma 1	73.0		77.8	b	88.1	b	87.4		87.0	b	88.5	b	99.2	b	67.1		*	
	Trichoderma 2	79.9		82.0	b	80.9	b	87.9		87.6	b	88.9	b	88.5	b	88.9		88.8	b
	P-Value	**0.3126**		**0.0100**		**0.0424**		**0.0596**		**0.0382**		**0.0107**		**0.0061**		**0.1562**		**0.0009**	
3 (15-30 cm)	Control	66.6	ns	76.8	b	79.0	b	79.8	s	80.9	s	76.8	b	73.4	b	71.8	b	73.0	b
	Compost	98.5		120.8	b	115.6	b	123.3		123.3		123.8	b	92.8	b	104.1	b	108.9	b
	Mycorrhiza	328.4		1176.5	a	1174.5	a	861.0		426.5		1112.3	a	946.3	a	1149.3	a	906.5	a
	Plant Extract	78.4		89.3	b	90.1	b	94.3		92.6		91.0	b	91.4	b	91.4	b	92.4	b
	Trichoderma & Amino Acids	94.1		91.5	b	93.0	b	95.8		101.1		98.2	b	89.5	b	97.1	b	101.6	b
	Trichoderma 1	63.7		73.4	b	80.9	b	77.5		78.7		76.1	b	67.5	b	75.3	b	*	
	Trichoderma 2	77.6		88.5	b	91.4	b	97.1		87.4		87.8	b	79.6	b	88.1	b	93.0	b
	P-Value	**0.6925**		**<.0001**		**<.0001**		**0.1205**		**0.1418**		**0.0006**		**0.0026**		**<.0001**		**0.0002**	
4 (0-15 cm)	Control	43.8	b	53.1	b	63.7	b	66.0	b	76.8	s	66.3	s	91.6	s	87.6	b	86.8	b
	Compost	69.2	b	74.7	b	87.4	b	92.0	b	91.7		97.3		119.8		131.3	b	127.4	b
	Mycorrhiza	1179.9	a	1397.8	a	1267.9	a	1307.2	a	841.2		718.5		652.9		859.3	a	1091.5	a
	Plant Extract	55.4	b	59.7	b	43.0	b	67.8	b	70.0		68.4		51.7		63.2	b	54.9	b
	Trichoderma & Amino Acids	62.8	b	78.4	b	76.0	b	73.3	b	68.8		90.2		94.7		88.0	b	87.8	b
	Trichoderma 1	82.1	b	77.6	b	80.0	b	83.6	b	82.0		80.9		57.6		71.0	b	*	
	Trichoderma 2	63.8	b	77.7	b	84.8	b	80.6	b	83.8		81.4		74.7		78.6	b	78.9	b
	P-Value	**0.0002**		**0.0128**		**0.0406**		**0.0172**		**0.0711**		**0.1024**		**0.2296**		**0.0385**		**0.021**	

[ns] Alphabetic letters show significant differences in values when significant differences occurred at a 5% confidence level (P<0.005)

Annexure C: Average root diameter (ARD) (mm) for 'Granny Smith'/M109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap! software for the biostimulant treatments at four soil depths (levels) during season 1 (2016/17). Level 1 (45 – 60 cm) below the surface, 2 (30 – 45 cm), 3 (15-30 cm) and 4 (0 – 15 cm). * Represents data not available.

Average Root Diameter (mm), Season 1 (201617)																			
Level	Treatment	15 December 2016		01 January 2017		15 February 2017		14 March 2017		19 April 2017		18 May 2017		12 July 2017		30 August 2017		02 October 2017	
1			n										n						
(45-60	Control	0.3855	s	0.4360	b	0.4409	b	0.5120	b	0.5036	b	0.4753	s	0.4710	b	0.4912	b	0.5010	b
cm)	Compost	0.6643		0.6090	b	0.6069	b	0.6160	b	0.6154	b	0.6168		0.5656	b	0.5945	b	0.5930	b
	Mycorrhiza	1.1959		7.7030	a	3.9811	a	4.2258	a	3.4972	a	2.7677		4.3898	a	4.5854	a	7.0820	a
	Plant Extract	0.5879		0.6150	b	0.6026	b	0.5386	b	0.5328	b	0.5302		0.5088	b	0.5113	b	0.5120	b
	Trichoderma & Amino Acids	0.6472		0.5720	b	0.5747	b	0.5473	b	0.5395	b	0.5399		0.5295	b	0.5326	b	0.5330	b
	Trichoderma 1	0.4988		0.4990	b	0.5268	b	0.5600	b	0.5620	b	0.5620		0.5046	b	0.5082	b	*	
	Trichoderma 2	0.4963		0.5300	b	0.5416	b	0.5467	b	0.5627	b	0.5615		0.5159	b	0.5362	b	0.5340	b
	P-Value	**0.7216**		**0.0001**		**0.0024**		**0.0013**		**<.0001**		**0.0828**		**0.0099**		**<.0001**		**0.015**	
2			n														n		
(30-45	Control	0.4900	s	0.5030	b	0.4897	b	0.4820	b	0.4810	b	0.4914	b	0.4280	b	0.4310	s	0.4390	b
cm)	Compost	0.6160		0.6314	b	0.6307	b	0.6070	b	0.6090	b	0.6095	b	0.6270	b	0.6520		0.6500	b
	Mycorrhiza	4.0780		4.6568	a	4.2528	a	4.6800	a	4.9680	a	4.7298	a	4.8500	a	3.7430		5.9960	a
	Plant Extract	0.5750		0.5202	b	0.5582	b	0.5160	b	0.5120	b	0.5030	b	0.5210	b	0.5170		0.5240	b
	Trichoderma & Amino Acids	0.5970		0.5326	b	0.5303	b	0.5500	b	0.5450	b	0.5395	b	0.5660	b	0.5690		0.5668	b
	Trichoderma 1	0.5120		0.5206	b	0.5850	b	0.6100	b	0.6050	b	0.6387	b	0.6480	b	0.4340		*	
	Trichoderma 2	0.5770		0.5524	b	0.5491	b	0.5710	b	0.6250	b	0.6286	b	0.6050	b	0.6290		0.6283	b
	P-Value	**0.2785**		**0.0025**		**0.0073**		**0.0149**		**0.0091**		**0.0027**		**0.0067**		**0.0576**		**0.0002**	
3			n						n		n								
(15-30	Control	0.4308	s	0.5179	b	0.5403	b	0.5737	s	0.5796	s	0.5847	b	0.5331	b	0.5260	b	0.5280	b
cm)	Compost	0.5817		0.6725	b	0.6683	b	0.6701		0.6647		0.6814	b	0.6761	b	0.6828	b	0.6860	b
	Mycorrhiza	1.9182		5.5970	a	6.1341	a	3.4304		1.8011		5.5258	a	5.1281	a	5.7999	a	5.3050	a
	Plant Extract	0.4957		0.6228	b	0.5936	b	0.5947		0.5825		0.5831	b	0.5812	b	0.5754	b	0.5810	b
	Trichoderma & Amino Acids	0.5570		0.5136	b	0.5146	b	0.5468		0.5917		0.5567	b	0.5431	b	0.5672	b	0.5730	b
	Trichoderma 1	0.4840		0.5494	b	0.5886	b	0.5946		0.5980		0.5918	b	0.5828	b	0.5944	b	*	
	Trichoderma 2	0.5632		0.5402	b	0.5591	b	0.5782		0.5813		0.5791	b	0.6246	b	0.6029	b	0.6110	b
	P-Value	**0.6923**		**<.0001**		**<.0001**		**0.0916**		**0.3422**		**<.0001**		**0.0006**		**<.0001**		**0.0038**	
4													n		n				
(0-15 cm)	Control	0.4451	b	0.4468	b	0.5320	b	0.5230	b	0.6100	b	0.5250	s	0.6400	s	0.6285	b	0.6280	b
	Compost	0.5980	b	0.6208	b	0.7430	b	0.7240	b	0.7190	b	0.7670		0.8650		0.8783	b	0.8590	b
	Mycorrhiza	4.7465	a	6.4266	a	5.6080	a	5.2610	a	5.5370	a	4.5410		3.7390		4.7649	a	5.6040	a
	Plant Extract	0.4550	b	0.4574	b	0.5100	b	0.5680	b	0.5410	b	0.5390		0.4770		0.5482	b	0.5300	b
	Trichoderma & Amino Acids	0.6308	b	0.5653	b	0.5610	b	0.5640	b	0.6080	b	0.7190		0.6580		0.6528	b	0.6530	b
	Trichoderma 1	0.6151	b	0.5753	b	0.5780	b	0.5970	b	0.5920	b	0.5880		0.5280		0.5672	b	*	
	Trichoderma 2	0.5147	b	0.6138	b	0.6180	b	0.6210	b	0.6830	b	0.6700		0.6220		0.6089	b	0.6060	b
	P-Value	**<.0001**		**0.0002**		**0.0086**		**0.0191**		**0.0342**		**0.0529**		**0.2011**		**0.0011**		**0.0034**	

[ns] Alphabetic letters show significant differences in values when significant differences occurred at a 5% confidence level (P<0.005)

Annexure D: Average root area (ARA) (mm^2) for 'Granny Smith'/M109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap! software for the biostimulant treatments at four soil depths (levels) during season 2 (2017)/18. Level 1 (45 – 60 cm) below the surface, 2 (30 – 45 cm), 3 (15-30 cm) and 4 (0 – 15 cm). * Represents data not available.

		Average Root Area (mm^2), Season 2 (2017/18)									
Level	**Treatment**	**28 November 2017**	**05 January 2018**	**31 January 2018**	**27 February 2018**	**11 March 2018**	**11 April 2018**	**23 May 2018**	**20 June 2018**	**02 August 2018**	**31 August 2018**
1 (45-60cm)	Control	53.9 ns	59.7 ns	63.8 ns	64.6 ns	51.7 ns	76.7 ns	68.1 ns	68.6 ns	77.0 ns	76.9 ns
	Compost	105.6	106.7	133.4	147.2	152.6	123.3	142.7	142.8	144.8	172.8
	Plant Extract	55.6	58.0	46.9	45.8	*	62.3	66.5	66.5	66.5	66.6
	P-Value	**0.2940**	**0.2551**	**0.0647**	**0.0844**	**0.5548**	**0.1600**	**0.1331**	**0.1325**	**0.1830**	**0.0814**
2 (30-45 cm)	Control	73.8 ns	72.5 ns	73.2 ns	73.7 ns	72.8 ns	76.8 b	73.5 ns	72.7 ns	77.1 ns	77.1 ns
	Compost	94.3	99.7	163.6	174.3	107.0	225.5 a	141.7	142.9	143.1	143.1
	Plant Extract	83.4	67.2	73.3	74.5	*	68.8 b	68.9	68.9	69.4	69.4
	P-Value	**0.2162**	**0.2027**	**0.2167**	**0.2294**	**0.1258**	**0.0033**	**0.0824**	**0.0776**	**0.1335**	**0.1335**
3 (15-30 cm)	Control	70.7 ns	79.0 ns	80.6 ns	81.0 ns	83.0 ns	82.0 ns	82.3 ns	82.9 ns	82.2 ns	82.2 ns
	Compost	92.0	101.1	316.4	174.6	171.4	*	175.6	176.0	176.0	178.5
	Plant Extract	54.4	78.3	80.1	80.9	*	95.6	97.3	97.3	97.3	97.3
	P-Value	**0.4052**	**0.6824**	**0.3337**	**0.1857**	**0.5031**	**0.4161**	**0.1787**	**0.1912**	**0.2769**	**0.2597**
4 (0-15 cm)	Control	89.7 ns	110.8 ns	108.5 ns	110.8 ns	112.0 ns	* ns	62.3 ns	64.7 ns	67.9 ns	69.5 ns
	Compost	172.8	145.1	141.9	124.4	*	*	142.2	144.9	135.1	145.9
	Plant Extract	50.1	89.5	87.2	95.2	*	96.5	86.0	86.0	86.0	55.0
	P-Value	**0.2493**	**0.3633**	**0.0908**	**0.4160**	**0.0000**	**0.1053**	**0.4121**	**0.4011**	**0.5729**	**0.3593**

ns Alphabetic letters show significant differences in values when significant differences occurred at a 5% confidence level (P<0.005)

Annexure E: Average root diameter (ARD) (mm) for 'Granny Smith'/M109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap! software for the biostimulant treatments at four soil depths (levels) during season 2 (2017)/18. Level 1 (45 – 60 cm) below the surface, 2 (30 – 45 cm), 3 (15-30 cm) and 4 (0 – 15 cm). * Represents data not available.

		Average Root Diameter (mm), Season 2 (2017/18)									
Level	**Treatment**	**28 November 2017**	**05 January 2018**	**31 January 2018**	**27 February 2018**	**11 March 2018**	**11 April 2018**	**23 May 2018**	**20 June 2018**	**02 August 2018**	**31 August 2018**
1 (45-60 cm)	Control	0.4918 [ns]	0.5079 [n s]	0.5016 [n s]	0.5004 [n s]	0.5133 [n s]	0.4861 [n s]	0.4970 [n s]	0.4982 [n s]	0.4922 [n s]	0.4919 [n s]
	Compost	0.6135	0.6123	0.7330	0.7752	0.8121	0.6276	0.7464	0.7445	0.7485	0.8386
	Plant Extract	0.5907	0.5238	0.4942	0.4782	*	0.4824	0.4897	0.4876	0.4876	0.4877
	P-Value	**0.2078**	**0.3357**	**0.1200**	**0.1246**	**0.6118**	**0.2399**	**0.1676**	**0.1715**	**0.2243**	**0.1414**
2 (30-45 cm)	Control	0.4341 [ns]	0.4421 [b]	0.4705 [n s]	0.4716 [n s]	0.4969 [n s]	0.4531 [b]	0.4647 [b]	0.4722 [b]	0.4649 [b]	0.4769 [b]
	Compost	0.6116	0.6279 [a]	0.7172	0.6839	0.6177	0.8294 [a]	0.6698 [a]	0.6804 [a]	0.6842 [a]	0.6842 [a]
	Plant Extract	0.5262	0.4856 [b]	0.5563	0.5573	*	0.4882 [b]	0.4880 [b]	0.4858 [b]	0.4858 [b]	0.4858 [b]
	P-Value	**0.0867**	**0.0398**	**0.1173**	**0.0851**	**0.0747**	**0.0142**	**0.0263**	**0.0092**	**0.0192**	**0.0179**
3 (30-45 cm)	Control	0.4874 [ns]	0.5490 [n s]	0.5640 [n s]	0.5665 [b]	0.6396 [n s]	0.5282 [n s]	0.5655 [b]	0.5758 [b]	0.5280 [b]	0.5280 [b]
	Compost	0.6197	0.6657	0.9075	0.8237 [a]	0.8181	0.0000 [n s]	0.8315 [a]	0.8856 [a]	0.8856 [a]	0.8856 [a]
	Plant Extract	0.4067	0.5740	0.5966	0.5976 [b]	*	0.6036 [n s]	0.6030 [b]	0.6030 [b]	0.6030 [a b]	0.6030 [a b]
	P-Value	**0.0763**	**0.5945**	**0.1187**	**0.0426**	**0.0822**	**0.5689**	**0.0391**	**0.0422**	**0.0463**	**0.0463**
4 (0-15 cm)	Control	0.5511 [ns]	0.6597 [n s]	0.6522 [b]	0.6650 [b]	*	0.6702 [n s]	0.5238 [n s]	0.5341 [n s]	0.5093 [n s]	0.5194 [n s]
	Compost	1.1768	0.9721	0.9846 [a]	1.0182 [a]	*	*	1.0050	0.9898	0.9488	0.9490
	Plant Extract	0.3077	0.6312	0.6219 [b]	0.6333 [b]	*	0.6273 [n s]	0.4141	0.4141	0.4141	0.2054
	P-Value	**0.0856**	**0.1693**	**0.0216**	**0.0412**		**0.6450**	**0.0818**	**0.0876**	**0.1422**	**0.0583**

[ns] Alphabetic letters show significant differences in values when significant differences occurred at a 5% confidence level (P<0.005)

Annexure F: Average root length (ARL) (mm) for 'Granny Smith'/M109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap! software for the biostimulant treatments at four soil depths (levels) during season 2 (2017)/18. Level 1 (45 – 60 cm) below the surface, 2 (30 – 45 cm), 3 (15-30 cm) and 4 (0 – 15 cm). * Represents data not available.

		Average Root Length (mm), Season 2 (2017/18)									
Level	**Treatmet**	**28 November 2017**	**05 January 2018**	**31 January 2018**	**27 February 2018**	**11 March 2018**	**11 April 2018**	**23 May 2018**	**20 June 2018**	**02 August 2018**	**31 August 2018**
1 (45-60 cm)	Control	35.25 ns	37.21 ns	40.07 ns	40.57 ns	34.47 ns	47.30 ns	42.98 ns	43.21 ns	47.64 ns	47.62 ns
	Compost	53.67	54.38	52.06	53.25	50.93	60.32	54.21	54.58	54.91	57.16
	Plant Extract	31.34	36.55	30.19	30.23	*	41.44	43.46	43.59	43.59	43.65
	P-Value	**0.4192**	**0.3636**	**0.1832**	**0.1305**	**0.1180**	**0.2254**	**0.2009**	**0.1945**	**0.2368**	**0.2498**
2 (30-45 cm)	Control	53.10 ns	51.70 ns	51.24 ns	51.49 ns	52.06 ns	53.06 b	51.56 ns	50.39 ns	52.78 ns	52.34 ns
	Compost	48.94	50.10	68.82	76.49	54.85	88.82 a	65.41	65.45	65.35	65.35
	Plant Extract	51.79	43.76	42.47	43.15	*	45.35 b	45.43	45.64	46.02	46.02
	P-Value	**0.3627**	**0.4688**	**0.2791**	**0.3098**	**0.3642**	**0.0154**	**0.1345**	**0.1602**	**0.2411**	**0.2401**
3 (15-30 cm)	Control	44.84 ns	45.83 ns	45.71 ns	45.68 ns	41.15 ns	49.06 ns	46.39 ns	46.64 ns	49.24 ns	49.24 ns
	Compost	46.39	46.98	94.65	65.11	64.27	*	65.46	64.98	64.98	65.18
	Plant Extract	41.08	40.80	43.64	43.83	*	52.46	53.72	53.72	53.72	53.72
	P-Value	**0.8404**	**0.6964**	**0.3910**	**0.3822**	**0.5589**	**0.7428**	**0.5000**	**0.5500**	**0.7048**	**0.6972**
4 (0-15 cm)	Control	48.74 ns	51.72 ns	51.24 ns	51.59 ns	*	52.63 ns	37.46 ns	38.37 ns	42.61 ns	43.33 ns
	Compost	50.28	48.98	47.15	42.71	*	*	43.36	44.50	43.25	44.31
	Plant Extract	25.94	45.16	45.31	48.52	*	49.30	37.72	37.72	37.72	23.17
	P-Value	**0.4871**	**0.6917**	**0.4307**	**0.2485**		**0.4373**	**0.9410**	**0.9253**	**0.9582**	**0.5840**

ns Alphabetic letters show significant differences in values when significant differences occurred at a 5% confidence level (P<0.005)

Annexure G: Average root volume (ARV) (mm^3) for 'Granny Smith'/M109 trees at Lovenstein, Vyeboom, quantified with a minirhizotron (CID-600) and Rootsnap! software for the biostimulant treatments at four soil depths (levels) during season 2 (2017)/18. Level 1 (45 – 60 cm) below the surface, 2 (30 – 45 cm), 3 (15-30 cm) and 4 (0 – 15 cm). * Represents data not available.

		Average Root Volume (mm^3), Season 2 (2017/18)									
Level	**Treatment**	**28 November 2017**	**05 January 2018**	**31 January 2018**	**27 February 2018**	**11 March 2018**	**11 April 2018**	**23 May 2018**	**20 June 2018**	**02 August 2018**	**31 August 2018**
1 (45-60 cm)	Control	7.24 ns	9.05 ns	9.30 ns	9.39 ns	6.60 ns	11.38 ns	9.79 ns	9.87 ns	11.39 ns	11.34 ns
	Compost	19.39	19.62	35.48	44.32	50.57	23.19	41.30	41.22	41.92	55.57
	Plant Exctract	9.13	8.16	6.35	6.03	*	8.01	8.67	8.64	8.64	8.65
	P-Value	**0.2582**	**0.2628**	**0.1483**	**0.2026**	**0.6230**	**0.1282**	**0.2452**	**0.2474**	**0.3142**	**0.1779**
2 (30-45 cm)	Control	9.07 ns	9.02 ns	9.48 ns	9.51 ns	8.70 ns	9.96 b	9.32 ns	9.38 ns	10.28 ns	10.42 ns
	Compost	16.95	18.23	33.59	35.52	18.83	50.08 a	27.66	28.22	28.40	28.40
	Plant Exctract	12.04	9.32	11.69	11.85	*	9.62 b	9.59	9.52	9.58	9.58
	P-Value	**0.0634**	**0.0544**	**0.1925**	**0.2027**	**0.0824**	**0.0012**	**0.0864**	**0.0649**	**0.1124**	**0.1129**
3 (15-30 cm)	Control	9.61 ns	12.78 ns	13.38 ns	13.49 b	16.64 ns	12.14 ns	13.62 b	14.48 b b	12.14 ns	12.14 ns
	Compost	15.92	19.24	93.27	44.74 a	43.18	0.00	44.68 a	46.5 a a	46.50	48.97
	Plant Exctract	6.20	15.14	14.86	15.12 b	*	16.55	16.71 b	16.71 b b	16.71	16.71
	P-Value	**0.3182**	**0.7321**	**0.2897**	**0.0474**	**0.3406**	**0.5148**	**0.0384**	**0.0371**	**0.0610**	**0.0539**
4 (0-15 cm)	Control	14.34 ns	21.08 ns	20.37 ns	21.01 ns	*	21.09 ns	8.77 ns	9.31 ns	8.90 ns	9.22 ns
	Compost	68.54	51.92	44.81	40.25	*	2	64.85	64.96	58.07	66.84
	Plant Exctract	9.57	16.68	15.38	17.06	*	17.36	19.54	19.54	19.54	12.34
	P-Value	**0.2302**	**0.3135**	**0.1219**	**0.2749**		**0.3175**	**0.2159**	**0.2181**	**0.2926**	**0.2685**

ns Alphabetic letters show significant differences in values when significant differences occurred at a 5% confidence level (P<0.005)